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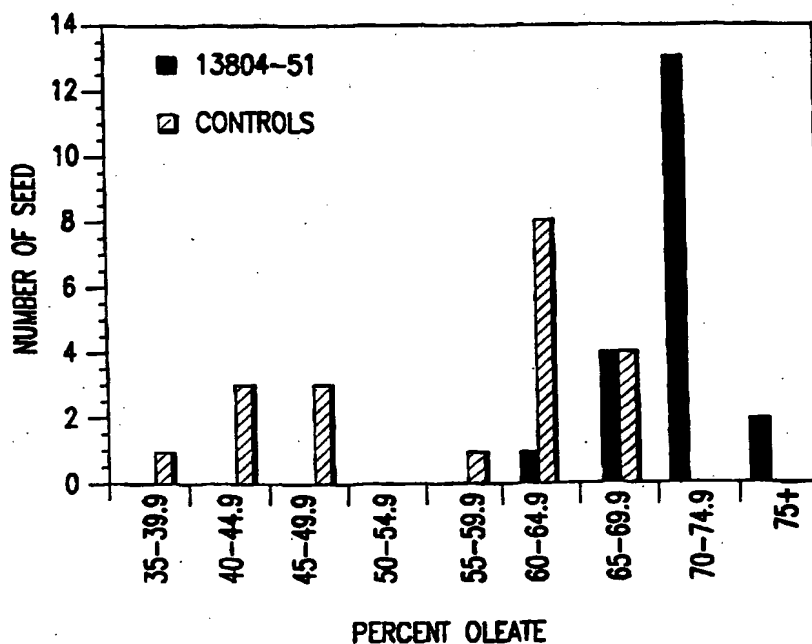
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(71) Applicants (for all designated States except US): MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US). MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).											
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(75) Inventors/Applicants (for US only): GIBSON, Susan, Irma [US/US]; Department of Biochemistry and Cellular Biology, Rice University, Houston, TX 77251 (US).											

(54) Title: **ALTERED LINOLENIC AND LINOLEIC ACID CONTENT IN PLANTS**



(57) Abstract

Transformed plants which have increased or decreased linolenic acid content are disclosed. Also disclosed are plants which express a linoleic acid desaturase gene.

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ALTERED LINOLENIC AND LINOLEIC ACID
CONTENT IN PLANTS

This is a continuation-in-part of U.S. Serial No. 08/156,551 filed November 22, 1993, which is a continuation of U.S. Serial No. 5 08/014,431, filed on February 5, 1993. The present invention relates to genetically engineered plants. In particular it relates to genetically engineered plants and seeds which have altered linolenic and linoleic acid content compared with naturally occurring plants.

BACKGROUND

10 Many crop species produce seed oils in which the fatty acid composition is not ideally suited to the intended use. The application of conventional breeding methods, coupled in some cases with mutagenesis, has resulted in the production of new varieties of several species with desirable alterations in the fatty acid composition of seed oil. A notable 15 example is the development of low erucic acid varieties of rapeseed (Stefansson 1983). Similar efforts have resulted in the reduction of the level of polyunsaturated 18-carbon fatty acids in soybean (Wilcox and Cavins 1985; Graef et al. 1988), sunflower (Fick 1989), and linseed oils (Green and Marshal 1984).

20 Most of the genetic variation in seed lipid fatty acid composition appears to involve the presence of an allele of a gene that disrupts normal fatty acid metabolism and leads to an accumulation of intermediate fatty acid products in the seed storage lipids (Downey 1987). However, it seems likely that, because of the inherent limitations of this 25 approach, many other desirable changes in seed oil fatty acid composition may require the directed application of genetic engineering methods.

α -Linolenic acid (18:3^{Δ9,12,15}) is an eighteen carbon fatty acid containing three *cis* double bonds at the 9-10, 12-13 and 15-16 carbons. It is found in the cells of higher plants as a constituent of cell membranes. It

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is also found in storage organs, such as in seeds. There it is designated oil bodies which are bounded by an electron dense structure that is thought to be a half-unit membrane and dispersed in the cytoplasmic environment of cells. When present as a constituent of cell membranes, linolenic acid is usually esterified to the sn-1 or sn-2 position of the glycerol moiety of a diacyl-glycerolipid. By contrast, when present in oil bodies, linolenic acid is usually esterified to the sn-1, sn-2 or sn-3 position of a triacylglycerolipid (TAG).

Linolenic acid is extensively used in the paint and varnish industry in view of its rapid oxidation. Flax seed is a predominant source of this oil. Soybean seed, on the other hand, does not have sufficient linolenic acid content to be used in this industry. Thus, increasing the linolenic acid content in a plant such as soybean would permit the use of the soybean oil in the paint and varnish industry.

On the other hand, it is undesirable to have significant levels of linolenic acid in cooking oils and foods. Linolenic acid is unstable during cooking and is rapidly oxidized. The oxidized products impart rancidity to the finished product. A rapeseed or soybean oil with reduced linolenic acid, such as containing 2% or less of linolenic acid, would be ideal for use as a cooking oil.

Linolenic acid is also a precursor in the biosynthesis of jasmonic acid, an important plant growth regulator. Linolenic acid is converted to jasmonic acid by introduction of an oxygen to the carbon chain by a lipoxygenase, followed by dehydration, reduction, and several β -oxidations (Vick and Zimmerman, 1984). The activity of jasmonic acid has been measured in terms of induction of pathogen defense responses. By application of free linolenic acid to plants, plant pathogen defenses can also be induced (Farmer and Ryan, 1992).

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A model has been proposed to explain the ability of free linolenic acid to exhibit the effects associated with jasmonic acid (Farmer and Ryan, 1992). It is hypothesized that all of the enzymatic activities which are required for the conversion of linolenic acid to jasmonic acid are
5 constitutively present in the cell and the rate limiting step in the production of jasmonic acid is the availability of free linolenic acid. A likely route for the production of the free linolenic acid is by the activity of a lipase in the plasma membrane.

It has been observed that exogenous jasmonic acid can more
10 powerfully activate defense responses than can wounding. This suggests that wounds cannot generate enough free linolenic acid to support high level production of jasmonic acid. The activity of the lipase or the availability of appropriate substrate for the lipase may be rate limiting upon wounding. Thus, increasing the linolenic acid content of plasma membrane may
15 positively influence "signal transduction" in plants and result in better protection against environment and pathogen stress.

Linolenic acid, as well as oleic and linoleic acids are also important constituents, as well as precursors of volatile carbonyl compounds, which contribute to the aroma of both fresh and cooked foods.
20 The major fatty acids of tomato fruit pericarp are oleic, linoleic and linolenic acids. As the fruit ripens, the levels of the latter two fatty acids decline resulting in the production of a number of 4-6 carbon containing aldehydes and ketones. One particular metabolite, *cis*-3-hexanol, has been shown to be present in higher levels in vine-ripened tomatoes compared to
25 supermarket tomatoes or tomatoes stored in refrigerators. It is likely, therefore, that the "aroma" of fresh fruits and vegetables can be "modulated" by regulation of the content of linolenic and linoleic acids, important substrates for the enzyme lipoxygenase and subsequently the

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hydroperoxide cleaving enzyme, which generates the volatile "aroma" compounds.

From the above, it is clear that the ability to vary the content of linolenic acid in plants would be desirable. However, to achieve this result it is necessary to determine what controls the product of linolenic acid in plants.

A large body of experimental evidence derived from radiochemical tracer studies has indicated that α -linolenic acid is synthesized by the desaturation of linoleic acid (18:2^{Δ9,12}) (reviewed in Harwood 1988;). However, the actual substrate for desaturation is not known.

In vivo and *in vitro* labelling studies suggest that there are possibly two distinct pathways for the synthesis of linolenic acid (Browse and Somerville, 1991). One possible pathway is thought to be located in the endoplasmic reticulum where linoleic acid esterified to the sn-2 position of phosphatidylcholine is a substrate for desaturation. However, the available evidence does not exclude the possibility that linoleic acid esterified to other lipids may also be a substrate.

A second possible pathway of linoleic acid desaturation is located in the plastid where the available evidence suggests that linoleic acid esterified to monogalactosyldiacylglycerol and, possibly, other plastid lipids is the substrate for desaturation.

Relatively little direct information is available concerning the enzymes involved in linoleic acid desaturation. Low levels of enzyme activity have been detected in microsomal membrane preparations from developing linseed (*Linum ussitatum*) (Browse and Slack, 1981) and, more recently, in preparations of gently lysed chloroplasts (Schmidt and Heinz, 1990a,b). The general features of the enzyme may be inferred from information available about other enzymes of this class.

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The most thoroughly characterized desaturase is the stearoyl-Coenzyme A (CoA) desaturase from vertebrate liver (reviewed by Holloway, 1983). This enzyme has been shown to be an integral membrane protein which contains non-heme iron. The desaturase reaction requires
5 fatty acyl-CoA, molecular oxygen and reduced cytochrome b5, another membrane protein. *In vivo*, the reduced cytochrome b5 is produced by the transfer of reducing equivalents from NADH via the activity of cytochrome b5 reductase, a flavin containing membrane protein.

The most thoroughly characterized desaturase from plants is
10 the stearoyl-ACP desaturase (McKeon and Stumpf, 1982; Shanklin and Somerville, 1991). This enzyme also requires molecular oxygen and a high potential reductant. However, in contrast to the animal enzyme, this desaturase is a soluble plastid protein which preferentially acts on a fatty acid esterified to acyl carrier protein (ACP) rather than CoA. This enzyme
15 also differs from the animal enzyme by utilizing reduced ferredoxin as an intermediate electron donor.

Other plant desaturases appear to be membrane proteins. The microsomal $\Delta 12$ oleate desaturase from several plant species has been assayed in membrane preparations from several plants (Harwood, 1988).
20 As with the stearoyl-CoA desaturase from animals, this enzyme requires molecular oxygen and reduced cytochrome b5 as an electron donor (Kearns et al., 1991). However, it appears that oleate esterified to a phospholipid is the substrate rather than a CoA ester.

With regard to the activity responsible for the making of
25 linolenic acid, little was known as to its source or origin. However, evidence that the amount of linolenic acid is related to the amount of linoleic acid desaturase activity has been obtained by analysis of the properties of the *fad3* mutant of *Arabidopsis thaliana* (Lemieux et al. 1990). This mutant is deficient in linolenic acid in the storage oils of its seed lipids and in the

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membrane lipids of different tissues to varying degrees. The mutant also had an increase in the amount of linoleic acid. This can be interpreted as evidence that the mutant is defective in the activity of a desaturase which converts linoleic acid to linolenic acid.

5 There is further evidence to suggest that the activity of this desaturase could be rate limiting for linolenic acid synthesis under normal circumstances. This was discovered by measuring the effects on fatty acid composition in heterozygous plants (i.e., *fad3*⁺/*fad*⁻) formed by crossing the wild type with the *fad3* mutant. In these F1 plants, which have one copy of
10 the normal *fad3* gene product instead of the two normally found in the wild type, the amount of linolenic acid was almost exactly intermediate between that found in either parent. This suggests that the amount of linolenic acid is proportional to the amount of functional *fad3* gene product (Lemieux et al., 1990).

15 These results do not shed any light, however, on the nature of the *fad3* gene product or whether the observed effects in mutants are related to either a decrease in quantity of desaturase protein or desaturase activity due to a defective protein.

 Moreover, nothing is known with any degree of certainty
20 about the linoleic acid desaturase from plant microsomes. As noted above, very little is known about the microsomal desaturases except that they probably utilize reduced cytochrome b5 as intermediate electron donor and probably utilize lipids rather than CoA or ACP esters as substrates.

 Moreover, as in many other aspects of plant biology, the lack
25 of specific information about the biochemistry and regulation of lipid metabolism makes it difficult to predict how the introduction of one or a few genes might usefully alter seed lipid synthesis.

 An additional problem arises from the fact that many of the key enzymes of lipid metabolism are membrane-bound and present in low

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quantities. Thus, attempts to solubilize and purify them from plant sources have not been successful.

SUMMARY OF THE INVENTION

The present invention provides structural coding sequences
5 encoding linoleic acid desaturase activity which can be used to alter the
linoleic and linolenic acid compositions of plants or to isolate other plant
linoleic acid desaturases. The present invention further provides a plant
capable of expressing a structural coding sequence to control the level of
linolenic acid or linoleic acid or both in the plant. The present invention
10 further provides a method for controlling the levels of linoleic and linolenic
acid in plants. It is also demonstrated by the present invention that the
linoleic acid desaturase enzyme activity in plant cells and tissues is a
controlling step in linolenic acid biosynthesis.

The present invention further relates to the engineering of two
15 advantageous traits into plants: increased and decreased α -linolenic acid
content in the structural lipids or storage oils of various crop plants.

In accomplishing the foregoing, there is provided, in
accordance with one aspect of the present invention, a genetically
transformed plant which has an elevated linolenic acid content comprising
20 a recombinant, double-stranded DNA molecule comprising

- (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;
- (ii) a structural coding sequence that causes the
25 production of an RNA sequence that encodes a linoleic
acid desaturase activity; and
- (iii) a 3' non-translated region that functions in plant
cells to promote polyadenylation to the 3' end of said RNA
sequence.

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In accordance with another aspect of the present invention, there is provided a genetically transformed plant which has a reduced linolenic acid content, comprising a recombinant, double-stranded DNA molecule comprising

- 5 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;
- (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least
10 a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and
- (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

15 There has also been provided, in accordance with another aspect of the present invention a method of producing a genetically transformed plant which has an elevated or reduced linolenic acid content. There has also been provided, in accordance with another aspect of the present invention a recombinant, double-stranded DNA molecule and plant cells
20 containing a recombinant, double-stranded DNA molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genetic map of the region of chromosome 2 of *Arabidopsis thaliana* where a linoleic acid desaturase gene is located and the identity of the yeast artificial chromosomes which carry this region of
25 the genome.

Figure 2 shows the structure of plasmid pBNDES3 which was obtained by inserting an EcoRI fragment containing the *B. napus* linoleic acid desaturase cDNA (fad3) into pBLUESCRIPT.

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Figure 3 shows the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) for the linoleic acid desaturase cDNA (fad3) from *B. napus*.

Figure 4 shows a comparison of the deduced amino acid sequence of one linoleic acid desaturase cDNA (fad3) from *B. napus* and the desA gene from *Synechocystis*. Identical residues are indicated by a solid box. Conservative substitutions are indicated by a stippled box.

Figure 5 shows the binary Ti plasmid vector pBI121.

Figure 6 shows the binary Ti plasmid pTiDES3 which was constructed by insertion of a linoleic acid desaturase cDNA (fad3) into pBI121.

Figure 7 shows the map of the plant transformation vector pMON13804.

Figure 8 shows the map of the plant transformation vector pMON13805.

Figure 9 shows the oil content of control and transformed canola seed in accordance with the present invention.

Figure 10 shows the nucleotide sequence (SEQ ID NO:9) for the linoleic acid desaturase cDNA (fadD) from *Arabidopsis*.

Figure 11 shows the deduced amino acid sequence (SEQ ID NO:10) for the linoleic acid desaturase cDNA (fadD) from *Arabidopsis*.

Figure 12 shows the nucleotide sequence (SEQ ID NO:11) for the linoleic acid desaturase cDNA (fadE) from *Arabidopsis*.

Figure 13 shows the deduced amino acid sequence (SEQ ID NO:12) for the linoleic acid desaturase cDNA (fadE) from *Arabidopsis*.

DETAILED DESCRIPTION OF THE INVENTION

A genetically transformed plant of the present invention which has an altered linolenic or linoleic acid content can be obtained by

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expressing the double-stranded DNA molecules described in this application.

The expression of a double-stranded DNA involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA.

Promoters

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA.

Any promoter which is known or is found to cause transcription of RNA in plant cells can be used in the present invention. Promoters which are useful in the present invention include any promoter that functions in a plant cell to cause the production of a RNA sequence. A number of promoters which are active in plant cells and are capable of producing a RNA sequence have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S and the figwort mosaic virus 35S-promoters, the light-inducible promoter from the small subunit of ribulose-1,5-bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), and the chlorophyll a/b binding protein gene promoter, etc. All of these promoters have been used to create various types of DNA constructs

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which have been expressed in plants; see, e.g., PCT publication WO 84/02913 (Rogers et al., Monsanto).

Promoters may be obtained from a variety of sources such as plants and plant viruses. Promoters can be used in the form that they
5 exist as isolated from plant genes such as ssRUBISCO genes, or can be modified to improve their effectiveness, such as with the enhanced CaMV35S promoter.

Those skilled in the art will recognize that the amount of linoleic acid desaturase needed to induce the desired alteration in linolenic acid
10 content may vary with the type of plant. It is also possible that extremes in linoleic acid desaturase activity may be deleterious to the plant. Therefore, in a preferred embodiment, promoter function should be optimized by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength and selecting a
15 transformant which produces the desired linoleic acid desaturase activity in the target tissues.

This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same
20 heterologous gene due to the site of gene insertion within the plant genome. (Commonly referred to as "position effect").

In a preferred embodiment, the promoters utilized in the double-stranded DNA molecules should have relatively high expression in tissues where the increased or decreased linolenic acid content is desired, such as
25 the seeds of the plant. In Canola, a particularly preferred promoter in this regard is the seed specific promoter described herein in greater detail in the accompanying examples.

In another preferred embodiment, the promoter used in the expression of the double-stranded DNA molecules of the present invention

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can be a constitutive promoter, expressing the DNA molecule in all or most of the tissues of the plant. However, the promoter selected for this embodiments should not cause expression at levels which are detrimental to plant health, growth and development.

5 β -conglycinin (also known as the 7S protein) is one of the major storage proteins in soybean (*Glycine max*) (Meinke et al., 1981). The 7S (β -conglycin) α' -subunit promoter, used in one aspect of this study to express the linoleic acid desaturase gene, has been shown to be both highly active and seed-specific (Doyle et al, 1986 and Beachy et al., 1985). The β -subunit
10 of β -conglycinin has been expressed, using its endogenous promoter, in the seeds of transgenic petunia and tobacco, showing that the promoter functions in a seed-specific manner in other plants (Bray et al., 1987). The promoter for β -conglycinin could be used to in accordance with the present invention. If used, this promoter could express the DNA molecule
15 specifically in seeds, which could lead to an alteration in the linolenic acid content of the seeds.

In addition, the endogenous plant linoleic acid desaturase promoters can be used in the present invention. These promoters should be useful in expressing a linoleic acid desaturase gene in specific tissues, such
20 as leaves, seeds or fruits. A number of other promoters with seed-specific or seed-enhanced expression are known and are likely to be expressed in seeds, which are oil accumulating cells. For illustration, the napin promoter and the acyl carrier protein promoters have been utilized in the modification of seed oil by antisense expression (Knutson et al., 1992).

25 The linolenic acid content of root tissue can be increased by expressing a linoleic acid desaturase gene behind a promoter which is expressed in roots. The promoter from the acid chitinase gene (Samac et al., 1990) is known to function in root tissue and could be used to express the linoleic acid desaturase in root tissue. Expression in root tissue could

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also be accomplished by utilizing the root specific subdomains of the CaMV35S promoter that have been identified. (Benfey et al., 1989). The linolenic acid content of leaf tissue can be increased by expressing the linoleic acid desaturase gene using a leaf active promoter such as
5 ssRUBISCO promoter or chlorophyll a/b binding protein gene promoter.

The linolenic acid content of fruits can be increased by expressing a linolenic acid desaturase gene behind a promoter which is functional in fruits. Such promoters could be either expressed at all developmental stages of the fruit or restricted to specific stages,
10 particularly fruit ripening.

The RNA produced by a DNA construct of the present invention can also contain a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5'
15 non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in the following examples, wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the
20 non-translated leader sequence can be derived from an unrelated promoter or coding sequence as discussed above.

Linoleic Acid Desaturase Structural Coding Sequences

The structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity can be the
25 sequences disclosed in the present application, or any sequence that can be obtained using the sequences disclosed in the present application, or any sequence that can be isolated using the method disclosed in the present application.

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The structural coding sequence can also be a part of or from the structural coding sequences disclosed in the present invention. It is possible that the active part of the linoleic acid desaturase is formed using only part of the structural coding sequences disclosed in the present application.

5 The structural coding sequences can be obtained from a variety of sources, such as algae, bacteria or plants. Preferably, structural coding sequences obtained from plants are used in accordance with the present invention.

10 Since virtually nothing was known about the properties of the linoleic acid desaturase structural coding sequence prior to the present invention, the method used in the present invention to isolate the structural coding sequence was based on the concept of map based cloning. The essential concept in map based cloning is to use information about the genetic map position of a structural coding sequence to isolate the region of
15 the chromosome surrounding the structural coding sequence, and then to use the isolated DNA to complement a mutation in the structural coding sequence. This strategy has never previously been reported in the isolation of any plant gene.

20 In order to implement map based cloning of the linoleic acid desaturase, mutants of *Arabidopsis thaliana* (L.) deficient in linoleic acid desaturase activity were isolated by screening randomly chosen individuals from mutagenized populations of plants for individual plants with altered leaf or seed fatty acid composition. (Browse et al. 1985; Lemieux et al. 1990). By screening thousands of plants for altered fatty acid composition,
25 mutants with decreased amounts of linolenic acid and increased amounts of linoleic acid in leaf and seed lipids were isolated. Physiological and genetic analyses of these mutants indicated that they fell into three complementation groups designated fad3, fadD and fadE.

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The fad3 mutants had very reduced levels of linolenic acid in seeds and roots but had almost normal levels of linolenic acid in leaves. This effect was interpreted as evidence that the fad3 locus encoded a microsomal desaturase which was responsible for desaturation of linoleic acid to linolenic acid on lipids made by the pathway of lipid biosynthesis in the endoplasmic reticulum, designated the "eukaryotic pathway" (Lemieux et al. 1990). This pathway is mostly responsible for the synthesis of lipids in non-green tissues such as seeds and roots, but plays a secondary role in leaves and other green tissues. Thus, a mutation in the fad3 gene would not be expected to have a major effect on the desaturation of leaf lipids.

In contrast to the fad3 mutant, the fadD mutant had almost normal fatty acid composition of roots and seeds, but had a strong reduction in the amount of linolenic acid in leaf lipids, and a corresponding increase in the amount of linoleic acid. (Browse et al., 1986). Thus, this mutant had the properties expected of a mutant deficient in a linoleic acid desaturase from the prokaryotic pathway which is primarily responsible for the synthesis of lipids in green tissues.

An unusual property of the fadD mutants was that they were very deficient in linoleic acid content when grown at temperatures above about 22 °C but had almost normal fatty acid composition when grown at temperatures below about 18 °C (McCourt et al., 1987). Since it was very unlikely that several independently isolated mutations would all give rise to a temperature conditional phenotype, it was concluded that a second desaturase must be partially responsible for desaturating linoleic acid to linolenic acid in green tissues. Therefore, the fadD mutant was remutagenized with ethylmethane sulfonate, self-fertilized to produce a segregating population of mutagenized plants (designated the M2 generation), and this population was screened for a mutant which was deficient in linolenic acid in green tissues at low temperatures. A mutant

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with this property was isolated and the mutation responsible for this effect was designated the *fadE* locus (Somerville and Browse, unpublished).

Isolation of the Linoleic Acid Desaturase Gene from Canola

The following example was used to isolate the structural coding
5 sequence from the *fad3* region. The method described herein could equally have been used to isolate either the *fadD* or *fadE* region.

In order to approximately locate the *fad3* mutation of the genetic map of *Arabidopsis*, a sexual cross was made between the *fad3* mutant line BL1 and the multiply marked mutant line W1 (Hugly et al., 1991). The F1
10 hybrids from this cross were permitted to self-fertilize and the resulting F2 plants were scored for both the segregating genetic markers and the altered fatty acid composition. The results of this analysis indicated that the *fad3* mutation was located on chromosome 2 near the marker *erecta*. In order to obtain a more accurate map position by RFLP mapping, a second sexual
15 cross was made between the *fad3* mutant line BL1 and the Niederzenz race of *Arabidopsis*. The F1 progeny were permitted to self-fertilize to produce the F2 generation. 137 F2 plants were grown during 3 weeks at 22° C (100 $\mu\text{E}/\text{m}^2/\text{s}$) in order to produce fully expanded rosettes, and a few leaves (representing a total weight of 0.2-0.5 g per plant) were harvested
20 from each plant in order to prepare DNA from them.

The leaves were frozen in liquid nitrogen, and ground in dry ice, using a mortar and a pestle. For each sample, the frozen powder was transferred to a microfuge tube and an equal amount of 2 X CTAB buffer (2% cetyltrimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH 8,
25 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpolypyrrolidone (PVP) 40,000) was added. The tubes were left at room temperature for 5 min to allow the powder to thaw. The homogenate was extracted once with a mixture of chloroform-isoamyl alcohol (24:1, v/v), and 1/10 vol of 10 X CTAB (10 % CTAB, 0.7 M NaCl) buffer was added to the aqueous phase, which was then

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reextracted with an equal volume of chloroform isoamyl alcohol (24:1, v/v). The aqueous phase was transferred to a fresh microfuge tube and 1.5 vol of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8, 10 mM EDTA) was added. The DNA was allowed to precipitate for 12 hr at 4
5 degrees, and collected by centrifugation (5 min at 10 000g). The DNA was resuspended in 100 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 M NaCl, and 100 µg/ml RNase A and incubated at 50°C for 30 min. The DNA was precipitated by adding 2.2 vol of ethanol and incubating on ice for 20 min. The DNA was collected by centrifugation and the pellet was washed once
10 with 1 ml of 70% ethanol, dried under vacuum for 3 min and resuspended in 10 µl of distilled water. The DNA was stored at -20°C until use.

The 137 plants were grown to maturity and their seeds were collected individually. The fatty acid composition of 10 individual seeds from each of the F2 plants was measured as described by Browse et al
15 (1986) in order to score the fad3 phenotype of each plant. Each seed was incubated in 1 ml of 1N HCl in methanol for 1h at 80°C. The tubes were cooled to room temperature and 1 ml of 0.9 % NaCl plus 0.3 ml of hexane were added. The tubes were agitated by vortexing and the phases separated by centrifugation (300xg for 5 min). The hexane phase was saved,
20 evaporated under a stream of nitrogen, and the fatty acid methyl esters were dissolved in 50 µl hexane. An aliquot (2 µl) was injected onto the gas chromatograph and the fatty acid methyl esters separated and quantitated by flame ionization as described (Browse et al., 1986).

The DNA samples (1 µg) were then cut with the appropriate
25 restriction enzyme (EcoR1 for the marker # 220, Bgl2 for the marker ASA2) using a concentration of 1XKGB buffer (Sambrook et al, 1989), 5 units of the restriction endonuclease and 100 µg/ml BSA. The volume of each sample was 10 µl and the incubation was performed at 37 °C for 4 h. The fragments were resolved by agarose gel electrophoresis (0.8 % agarose

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in 1X TAE buffer; Sambrock et al., 1989) and transferred to nylon filters (hybond N+), using the alkaline transfer method as described by the manufacturer. The nylon filters were probed (according to Church and Gilbert, 1984) with radioactively labelled fragments of DNA (Sambrock et al., 1989) corresponding to known RFLP markers which had previously been mapped in the approximate vicinity of the *fad3* locus on chromosome 2. The RFLP markers 220 (Chang et al 1988) and ASA2 were found to map close to the *fad3* locus. Analysis of the pattern of recombinants (Table 1) indicated that both ASA2 and 220 were located on the same side of the *fad3* locus at distances of 0.4 and 2.2 centimorgans (cM), respectively.

Table 1

	<u># of plants</u>	<u>220</u>	<u>ASA2</u>	<u>fad3</u>
	67	H	H	+/-
15	30	L	L	-/-
	34	N	N	+/+
	3	H	N	+/+
	1	L	H	+/-
	1	N	H	+/-
20	1	H	H	-/-

Table 1 shows the genotype of the F2 plants used for mapping the *fad3* locus. L is for Landsberg (background of the *fad3* mutant), N is for Niederzenz, H for heterozygous. A total of 137 F2 plants were analyzed. The number of recombinant plants between *fad3* and 220 or ASA2 was 6 and 1 respectively.

In order to isolate the region of the chromosome containing the *fad3* locus, the RFLP markers 220 and ASA2 were used as hybridization probes to screen several yeast artificial chromosome (YAC) libraries. (Grill

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and Somerville, 1991; Ward and Jen, 1990). The YAC filters were prepared according to Grill and Somerville (1991). The library was replicated onto nylon filters disposed on petri dishes of SC -- (synthetic complete medium minus tryptophan and uracil; Sherman et al., 1986). The cells were allowed
5 to grow for 12 h at 30°C, and the filters were transferred for 15 min on a Whatman 3MM paper saturated with 1 M sorbitol, 50 mM DTT, 50 mM EDTA (pH 8).

The cell wall of the cells was then digested with lyticase, by incubating the filters on a Whatman paper saturated with 1M sorbitol, 50
10 mM EDTA and 2 mg/ml lyticase (Sigma Co., St. Louis, MO) for 12 h at 30°C. The filters were then transferred on a Whatman 3MM paper saturated with 0.5 M NaOH, 1.5 M NaCl for 15 min, neutralized with 0.5 M Tris-HCl pH 8 for 15 min and quickly rinsed in 2XSSC (SSC is 10mM sodium citrate, 150mM NaCl, pH 7). The filters were allowed to dry, and
15 were transferred to a vacuum oven at 80°C for 1 h. They were subsequently hybridized according to Church and Gilbert (1984), with probes labelled with ³²P according to Sambrook et al. (1989).

The DNA of RFLP probe 220 was prepared from 100 ml of liquid culture lysate using the lambdasorb procedure (Promega Corp., Madison,
20 WI); the cDNA encoding ASA2 was excised from the original plasmid (pKN140C; obtained from Dr. G. Fink, Whitehead Institute, Cambridge, MA) with Hind3 and cloned into the Hind3 site of pBLUESCRIPT. The plasmid DNA was then purified by Cesium chloride gradients according to Sambrook et al (1989), digested with Hind3 and the DNA insert was gel
25 purified twice by electroelution according to Sambrook et al (1989).

In order to probe the libraries, the whole DNA from RFLP220 was used as a hybridization probe. By contrast, only the DNA insert of ASA2 was used as a probe. The RFLP probe 220 hybridized to YAC

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EG4E8 and EG9D12. The probe ASA2 hybridized to YACs EW15G1, EW15B4 and EW7D11.

In order to determine if these YACs contained all of the DNA between RFLP220 and ASA2, small regions of DNA from the ends of the
5 inserts in EG4E8 and EW15G1 were prepared by inverse PCR (Grill and Somerville, 1991). For that purpose, DNA was prepared from the appropriate YAC clones. The clones (single colonies) were grown to saturation in SC-- liquid cultures, and 1 ml of these cultures was used to inoculate 40 ml liquid cultures (in SC-- medium) that were allowed to grow
10 for 16 h at 30°C. The cells were collected by centrifugation, washed once in 1 M sorbitol, 50 mM EDTA, resuspended in 200 µl of 1 M sorbitol, 50 mM EDTA, 100 mM sodium citrate pH 5.8, 2 mM β-mercaptoethanol and 2 mg/ml lyticase, and incubated 2 h at 30 °C.

Next, 350 µl of 2XCTAB buffer was added and the DNA was
15 purified as described above. DNA (5 µg) of each clone was digested separately with HincII, AluI, EcoRV and RsaI (in 1XKGB buffer, at 37 °C for 4 h; final volume: 50 µl). The reactions were stopped by heating at 65 °C for 15 min, extracted once with one volume of phenol saturated with TE pH 8, followed by an extraction with 1 volume of chloroform - isoamyl
20 alcohol mixture (24:1, vol/vol). The DNA was recovered by ethanol precipitation and resuspended in sterile distilled water. The ligation reactions were performed using 300 ng of DNA in a final volume of 50 µl. The reactions were carried out in 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM DTT, 1.2 mM ATP with 1 U of ligase, for 2 h at 20 °C, and stopped by
25 heating at 68 °C for 30 min.

The PCR reactions were carried out as follows: The buffers used were the ones indicated by the suppliers except for the Perkin Elmer enzyme for which the reaction was supplemented with an additional 1.4 mM MgCl₂ (final concentration 2.9 mM Mg). The dNTP final concentration

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was 125 μ M when the Perkin Elmer enzyme was used and 200 μ M with the Taq polymerases from other sources. In all cases, 100 ng of each oligonucleotide was used. The final volume was 100 μ l. When no product was obtained, the reactions were carried out again in the same conditions
5 except that formamide was added to a final concentration of 3 %.

The left end was amplified from the ligation products of the EcoRV and RsaI digests, using the oligonucleotides EG1 (GGCGATGCTGTCGGAATGGACGATA) (SEQ. ID NO. 3) and EG2 (CTTGGAGCCACTATCGACTACGCGATC) (SEQ. ID NO. 4).

10 The right end of the clones obtained from the EG library was amplified from the ligation products of the AluI and HincII digests, using the oligonucleotides EG3 (CCGATCTCAAGATTACGGAAT) (SEQ. ID NO. 5) and EG4 (TTCCTAATGCAGGAGTCGCATAAG) (SEQ. ID NO. 6).

The right end of the clones obtained from the EW YAC library
15 was amplified using the oligonucleotides H1 (AGGAGTCGCATAAGGGAG) (SEQ. ID NO. 7) and H2 (GGGAAGTGAATGGAGAC) (SEQ. ID NO. 8), using the same cycle conditions as above, except that the annealing temperature was reduced to 50 °C.

After the reactions were completed, 5 μ l of each mixture were
20 electrophoresed on an agarose gel to separate the amplification product from primers. The slice of agarose that contained the amplified band was excised from the gel and melted in 1 ml of distilled water. Large amounts of product could then be produced, by reamplification of 5 μ l of the melted slice. The PCR products were then purified by electroelution or by using
25 GeneClean (Bio101) and used as hybridization probes to probe filters containing the isolated YAC DNA restricted by several enzymes. The probe made from the right end of EW15G1 hybridized to EG4E8 and similarly, a probe from the right end of EG4E8 hybridized to EW15G1.

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Thus, it was concluded that the YACs EG4E8 and EW15G1 contained all of the DNA in the region of the chromosome between RFLP220 and ASA2.

The size of the YAC clones was estimated by field inversion electrophoresis (CHEF, Vollrath and Davis, 1987). High molecular weight
5 DNA was prepared as follows: the yeast cells which contained the YAC clones were grown and treated with lyticase as for preparing DNA as described above. The spheroplasts were then resuspended in an equal volume of 1M sorbitol, 50 mM EDTA, 1 % low melt agarose at 37°C. The mixture was poured in a mould (Biorad) which was set on ice to allow the
10 agarose to harden.

The resulting plugs were incubated for 12 h in 0.5 M EDTA pH 9, 1% lauryl sarcosine 1 mg/ml Proteinase K at 50°C. The plugs were subsequently washed twice in 50 mM EDTA and stored at 4°C until use. The CHEF gel was run in 1XTBE for 16 h at 200 V, with a switching
15 interval of 20 s; the temperature of the buffer was maintained at 14 °C during the run. The sizes of the YACs were determined by comparison with a lambda ladder and the yeast chromosomes, and were as follows: EG4E8, 90 kb; EG9D12, 190 kb; EW15G1, 90 kb; EW15B4, 70 kb, EW7D11, 125 kb. These sizes permitted us to roughly determine a correspondence
20 between physical and genetic distances: the distance that separates 220 from ASA2 cannot exceed 180 kb, the sum of the size of the 2 YACs EG4E8 and EW15G1. Since the corresponding genetic distance is 1.7 cM, one can roughly estimate that, in this particular cross and in this particular region of the genome, the value of 1 cM is close to 100kb. Thus, since the
25 fad3 gene maps only 0.4 cM away from ASA2, the corresponding physical distance should be close to 40 kb. We then concluded that fad3 was probably located on the YAC EW7D11, which is the largest YAC hybridizing with ASA2. See Figure 1.

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In order to test the possibility that the YAC EW7D11 carried the *fad3* gene, the YAC was used to probe a cDNA library made from developing seeds of Canola (*Brassica napus* L.). Even though the YAC was isolated from *Arabidopsis*, the fact that *Arabidopsis* and *B. napus* are both members of the family Cruciferae led us to predict that the homologous genes from these two species would be sufficiently identical at the nucleotide sequence level so that the *Arabidopsis* gene would hybridize to the *B. napus* gene. We also assumed that, because it catalyzes a chemically similar reaction to the stearyl-ACP desaturase, it would be expressed at similar moderately high levels in developing seeds (Shanklin and Somerville, 1991). Since EW7D11 contained only about 0.2% of the total genome, we expected it to contain only about 2 moderately abundantly expressed genes (i.e., genes in which the mRNA is between 0.1 and 0.01% of total mRNA).

DNA of YAC EW7D11 was isolated as follows: high molecular weight DNA was prepared from the yeast cells that contained the YAC EW7D11 as described above, and several preparative low-melt agarose CHEF gels were run in 1XTBC buffer (same as TBE except that CDTA was substituted for EDTA). The slices that contained the YAC were excised from the gels and pooled. Three slices were melted at 65°C and extracted with an equal volume of phenol saturated with TE. The aqueous phase was saved and reduced to 0.5 ml by repeated extractions with isobutyl alcohol. The remaining agarose was removed by several phenol extractions, followed by two chloroform-isoamyl alcohol extractions. The DNA was precipitated by adding 2 µg of linear acrylamide as a carrier plus 10 µl of 5M NaCl and 1.1 ml of ethanol, and incubating 20 min at 0 °C. The DNA pellet was recovered by centrifugation, washed in 70% ethanol, dried under vacuum and resuspended in 50 µl of distilled water. The DNA (50 ng) was radioactively labelled and used to probe a cDNA library in λgt11.

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The nitrocellulose filters were processed as described in Sambrook et al (1989). Duplicate filters were used, and the films were exposed 5-7 days in order to obtain a good signal. From among 200,000 plaques screened in this way, 31 hybridized to EW7D11. Among these 31 clones, 17 were homologous to each other, as checked by cross hybridization in stringent conditions. The size of the inserts in the 17 clones was estimated and the clone with the largest cDNA was retained for further analysis. A small scale preparation of this phage was prepared using the lambdasorb method, and the insert was excised by restricting with EcoRI. This insert was ligated into a pBLUESCRIPT II vector linearized with EcoRI, and the ligation mixture was used to transform *E. coli* strain DH5 α .

One of the recombinant clones was designated pBNDES3 (Figure 2), and retained for sequencing. The sequence was determined on both strands, using the sequenase enzyme, (US Biochemicals, Cleveland, OH) according to the instructions provided by the supplier. The nucleotide sequence of the insert in pBNDES3 is presented as Figure 3. The deduced amino acid sequence of the largest open reading frame in the nucleotide sequence is also shown in Figure 3.

Comparison of the deduced amino acid sequence of the 383 amino acid open reading frame in clone pBNDES3 against the known sequences in GenBank release 70 was performed using the FASTA program (Lipman and Pearson, 1985). This analysis revealed that the sequence from pBNDES3 had a region of significant homology to a previously characterized desaturase gene from the cyanobacterium *Synechocystis* (Figure 4). (Wada et al. 1990). This was considered suggestive evidence that the clone pBNDES3 encoded a desaturase which was probably the fad3 structural coding sequence product. This was subsequently confirmed by a genetic complementation experiment.

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The cDNA was cloned into plant transformation vector pBI121 (Figure 5) under the control of the CaMV35S promoter to construct pTiDES3 (Figure 6). Plasmid pTiDES3 was introduced into an *Agrobacterium tumefaciens* strain which also carried an Ri plasmid and this was used to produce transgenic rooty tumors from both wild type Arabidopsis and the fad3 mutant. Transgenic tissue was selected for antibiotic resistance to confirm the presence of the pTiDES3. Fatty acid methyl esters were then prepared and examined by gas chromatography to determine the profile of fatty acids being produced in the tissue. The levels of linolenic acid increased, demonstrating that the cDNA on pTiDES3 can complement the fad3 mutation. These results, which are described in detail in Example 1 below, confirm the identity of the cDNA as encoding a linoleic acid desaturase.

The isolation of a plant structural coding sequence provides those skilled in the art with a tool for the manipulation of gene expression by the mechanism of antisense RNA. The technique of antisense RNA is based upon introduction of a chimeric gene which will produce an RNA transcript that is complementary to a target gene (reviewed in Bird and Ray, 1991). The resulting phenotype is a reduction in the gene product from the endogenous gene. The portion of the gene which is sufficient for achieving the antisense effect is variable in that numerous fragments or combinations thereof are likely to be effective. Various portions of the structural coding sequence of linoleic acid desaturase isolated either from cDNA or genomic clones are likely capable of reducing linolenic acid levels in plants by reduction in levels of linoleic acid desaturase levels. An example of using an antisense oriented linoleic acid desaturase structural coding sequence is set out in Example 2.

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Polyadenylation Signal

The 3' non-translated region of the double stranded DNA molecule of the present invention contains a region that functions in plant cells to promote polyadenylation to the 3' end of the RNA sequence. Any
5 such regions can be used within the scope of the present invention. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylated signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and
10 (2) 3' regions of plant genes like the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that from the NOS gene, described in greater detail in the examples below.

Plant Transformation/Regeneration

Any plant which can be transformed to contain the double-
15 stranded DNA molecule of the present invention are included within the scope of this invention. Preferred plants which can be made to have increased or decreased linolenic acid content by practice of the present invention include, but are not limited to sunflower, safflower, cotton, corn, wheat, rice, peanut, canola/oilseed rape, barley, sorghum, soybean, flax,
20 tomato, almond, cashew and walnut.

A double-stranded DNA molecule of the present invention containing the functional plant linoleic acid desaturase gene can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of
25 *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1984), Klee (1985) and EPO publication 120,516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into

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plant cells. Such methods can involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using bacteria, viruses or pollen.

5 A plasmid expression vector, suitable for the expression of the linoleic acid desaturase gene in monocots is composed of the following: a promoter that is specific or enhanced for expression in the lipid storage tissues and a 3' polyadenylation sequence such as the nopaline synthase 3' sequence (NOS 3'; Fraley et al., 1983). This expression cassette may be
10 assembled on high copy replicons suitable for the production of large quantities of DNA.

A particularly useful *Agrobacterium*-based plant transformation vector for use in transformation of dicotyledonous plants is plasmid vector pMON530 (Rogers, S.G., 1987). Plasmid pMON530 (see Figure 7) is a
15 derivative of pMON505 prepared by transferring the 2.3 kb *Stu*I-*Hind*III fragment of pMON316 (Rogers, S.G., 1987) into pMON526. Plasmid pMON526 is a simple derivative of pMON505 in which the *Sma*I site is removed by digestion with *Xma*I, treatment with Klenow polymerase and ligation. Plasmid pMON530 retains all the properties of pMON505 and the
20 *CaMV*35S-NOS expression cassette and now contains a unique cleavage site for *Sma*I between the promoter and polyadenylation signal.

Vector pMON505 is a derivative of pMON200 (Rogers, S.G., 1987) in which the *Ti* plasmid homology region, LIH, has been replaced with a 3.8 kb *Hind*III to *Sma*I segment of the mini RK2 plasmid, pTJS75
25 (Schmidhauser & Helinski, 1985). This segment contains the RK2 origin of replication, *ori*V, and the origin of transfer, *ori*T, for conjugation into *Agrobacterium* using the tri-parental mating procedure (Horsch & Klee, 1986). Plasmid pMON505 retains all the important features of pMON200 including the synthetic multi-linker for insertion of desired DNA fragments,

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the chimeric NOS/NPTII/NOS gene for kanamycin resistance in plant cells, the spectinomycin/streptomycin resistance determinant for selection in *E. coli* and *A. tumefaciens*, an intact nopaline synthase gene for facile scoring of transformants and inheritance in progeny and a pBR322 origin of replication for ease in making large amounts of the vector in *E. coli*.
5 Plasmid pMON505 contains a single T-DNA border derived from the right end of the pTiT37 nopaline-type T-DNA. Southern analyses have shown that plasmid pMON505 and any DNA that it carries are integrated into the plant genome, that is, the entire plasmid is the T-DNA that is inserted into
10 the plant genome. One end of the integrated DNA is located between the right border sequence and the nopaline synthase gene and the other end is between the border sequence and the pBR322 sequences.

When adequate numbers of cells (or protoplasts) containing the linoleic acid desaturase gene are obtained, the cells (or protoplasts) are
15 regenerated into whole plants. Choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from *Leguminosae* (alfalfa, soybean, clover, etc.), *Umbelliferae* (carrot, celery, parsnip), *Cruciferae* (cabbage, radish, rapeseed, etc.), *Cucurbitaceae* (melons and cucumber), *Gramineae* (wheat, rice, corn, etc.), *Solanaceae*
20 (potato, tobacco, tomato, peppers) and various floral crops. See, e.g., Ammirato (1984); Shimamoto, 1989; Fromm, 1990; Vasil and Vasil, 1990.

Uses of Linoleic Acid Desaturase

The present invention can be used for any modification (either increase, decrease, or mere change) of the oil content of a plant or plant
25 tissue. Linolenic acid is an important constituent of several membranes in plant cells.

One preferred method is to modify the oil content of the plant to improve the plant's temperature sensitivity. For instance, plants deficient in linolenic acid display reduced fitness at low temperature (Hugly and

Somerville, 1992). Also, increased linoleic acid content in vegetative tissues has been implicated as a factor in freezing tolerance in higher plants (Steponkus et al., 1990 and references therein). In a preferred embodiment, expression of the linoleic acid desaturase structural coding
5 sequence can result in the genetic modification of higher plants to achieve tolerance to low environmental temperatures. Transformation with pTiDES3 demonstrates that linolenic acid levels can be increased by expression of this gene in a constitutive manner. Chilling or freezing injury in crops may be overcome by expression of this gene in vegetative or
10 reproductive tissues by employing an appropriate promoter.

Linolenic acid, a polyunsaturated fatty acid, is also extensively used in the paint and varnish industry in view of its rapid oxidation. Flax seed is a predominant source of this oil. Higher quantities of this fatty acid in rapeseed or soybean will provide opportunities for using vegetable oils
15 from these sources as a replacement for linseed (flax) oil. Expression of a linoleic acid desaturase structural coding sequence in seed tissue can result in a higher proportion of linolenic acid in the storage oil.

Linolenic acid is further a precursor in the biosynthesis of jasmonic acid, an important plant growth regulator. Linolenic acid is
20 converted to jasmonic acid by introduction of an oxygen to the carbon chain by a lipoxygenase, followed by dehydration, reduction, and several β -oxidations (Vick and Zimmerman, 1984). The activity of jasmonic acid has been measured in terms of induction of pathogen defense responses. By application of free linolenic acid to plants, plant pathogen defenses can also
25 be induced (Farmer and Ryan, 1992). A model has been proposed to explain the ability of free linolenic acid to exhibit the effects associated with jasmonic acid (Farmer and Ryan, 1992). It is hypothesized that all of the enzymatic activities which are required for the conversion of linolenic acid to jasmonic acid are constitutively present in the cell and the rate limiting

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step in the production of jasmonic acid is the availability of free linolenic acid. A likely route for the production of the free linolenic acid is by the activity of a lipase in the plasma membrane.

It further has been observed that exogenous jasmonic acid can
5 more powerfully activate defense responses than can wounding. This suggests that wounds cannot generate enough free linolenic acid to support high level production of jasmonic acid. The activity of the lipase or the availability of appropriate substrate for the lipase may be rate limiting upon wounding. By increasing levels of available substrate, increasing
10 linolenic acid levels in the plasma membrane, it should be possible to enhance a plant's ability to respond to pathogens by allowing for a higher production of jasmonic acid. Expression of a linoleic acid desaturase structural coding sequence can result in a higher molar percent linolenic acid in the plasma membrane of a plant cell therefore enhancing the
15 jasmonic acid signaling pathway. It is our intent to evaluate plants containing high linolenic acid levels in root and foliar tissues for their pathogen resistance.

It is also undesirable to have significant levels of linolenic acid in cooking oils. Linolenic acid is unstable during cooking and is rapidly oxidized.
20 The oxidized products impart rancidity to the finished product. Rapeseed or soybean oil containing less than about 3%, and preferably 2% or less of linolenic acid is ideal for use as a cooking oil. By expression of the antisense of the structural coding sequence for linoleic acid desaturase, it is possible to reduce the linolenic acid content of these oils.

25 All higher plants have linolenic acid and, therefore, contain genes for linoleic acid desaturases. Because of the many examples in which genes isolated from one plant species have been used to isolate the homologous genes from other plant species, it is apparent to any one skilled in the art, that the results presented here do not only pertain to the use of the *B.*

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napus *fad3* gene, or to the use of the gene to modify fatty acid composition in *B. napus*. Obviously, the linoleic acid desaturases from many organisms could be used to increase linolenic acid biosynthesis and accumulation in plants and enzymes from any other higher plant or algae can serve as
5 sources for linoleic acid desaturase genes. For example, since a YAC containing the *Arabidopsis* gene was used to isolate the *B. napus* gene, it is apparent that the insert in pBNDES3 could be used as a probe of genomic libraries for isolation of the corresponding full length genes from other plant species. It is also likely that the information contained in the sequence of
10 this gene will be useful to clone other lipid desaturases genes.

Expression of a linoleic acid desaturase in a sense orientation may also allow for the isolation of plants with reduced levels of linolenic acid. This could be accomplished by the mechanism of co-suppression (Bird and Ray, 1991). The molecular mechanism of co-suppression is at this
15 time poorly understood but occurs when plants are transformed with a gene that is identical or highly homologous to an allele found in the plants genome. There are several examples where expression of a chimeric gene in plants can result in a reduction of the gene product from both the chimeric gene and the endogenous gene(s). Those skilled in the art will recognize that
20 the resulting decrease in linolenic acid would be a direct result of expression of the linoleic acid desaturase structural coding sequence and would be correlated to the linoleic acid desaturase activity in the transformed plant.

Linolenic acid levels in plant cells can also be modified by isolating genes encoding transcription factors which interact with the
25 upstream regulatory elements of the plant linoleic acid desaturase gene(s). Enhanced expression of these transcription factors in plant cells can effect the expression of the linoleic acid desaturase gene. Under these conditions, the increased or decreased linolenic acid content would also be caused by a corresponding increase or decrease in the activity of the linoleic acid

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desaturase enzyme although the mechanism is different. Methods for the isolation of transcription factors have been described (Katagiri, 1989).

The following examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications, truncations, etc. can be made to the methods and genes described herein while not departing from the spirit and scope of the present invention.

Example 1

10 Expression of fad3 gene to increase linolenic acid

To verify the assumption that the cDNA insert in pBNDES3 encodes a linoleic acid desaturase, both wild type and fad3 mutation *Arabidopsis* were transformed to contain the cDNA insert. In order to express the linoleic acid desaturase structural coding sequence (hereafter referred to as the "fad3 gene") in plant cells, the plasmid pBNDES3 was digested with XhoI and the ends were filled in with the Klenow fragment of DNA polymerase (Sambrook et al 1989). The cDNA insert was subsequently excised by digestion with SacI and ligated into the SacI and SmaI sites of the binary Ti plasmid vector pBI121 (Clontech Laboratories), thereby replacing the GUS reading frame. The ligation reaction was carried out in 20 µl for 12 h at 16 °C using 100 ng of both insert and vector, and one unit of T4 DNA ligase. The ligation mixture was used to transform competent DH5α *E. coli* cells (prepared by the calcium chloride method, according to Sambrook et al, 1989), and transformants were selected on L-broth plates that contained 50 µg/µl Kanamycin. Alkaline minipreparations of recombinant clones were analyzed for the correct restriction pattern. One of these plasmids, designated pTiDES3, was used for further experiments.

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This plasmid was electroporated (according to Mersereau and Pazour, 1990) into *Agrobacterium tumefaciens* strain R1000 which carries an Ri plasmid. The transformed bacteria were selected on kanamycin LB plates for 2 days at 30 °C. DNA minipreparations of several recombinant
5 bacteria were performed and analyzed as described above to verify the presence of the construct.

Young flowering stems of wild type and the fad3 mutant of *Arabidopsis* were sterilized for 30 min in 10% commercial bleach, 0.02% Triton X100, and 2-cm explants that contained the flowering stem were
10 infected with R1000 (pTiDES3) This was performed by dipping the sectioned extremity in a drop of an overnight culture of the appropriate *Agrobacterium* that was grown from a single colony in LB medium supplemented with 50 ug/ml Kanamycin.

The infected stems were cultured for two days on solid MSO
15 medium (Gibco MS salts plus Gamborg B5 vitamins, 3% sucrose and 0.8% agar). At this time the stem segments were transferred for 5 weeks to MSO medium containing 200 µg/ml cefotaxime to kill the bacterium. After approximately two weeks, most of the stem explants had developed rooty tumors resulting from transfer of parts of the Ri plasmid into cells of the
20 stem explants. In order to identify the rooty tumors which had also received the binary Ti plasmid pTiDES3, approximately 24 rooty tumors from each treatment were transferred to MSO medium containing 50 µg/ml of kanamycin to select for the growth of those roots which had been cotransformed with the binary Ti plasmid; the medium contained also 200
25 µg/ml of cefotaxime to inhibit bacterial growth. Following a further period of growth for 2 weeks, fatty acid methyl esters were prepared (as described above) from the roots for analysis by gas chromatography. The results of these analyses are presented in Table 2.

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Table 2. Genotype

	mol% Fatty acid	wildtype pBI121	fad 3 pBI121	wildtype pTiDES3	fad3 pTiDES3
5					
	16:0	22.0±2.9	21.2±1.6	21.1±0.9	21.3±2.3
	16:1	2.5±0.7	1.6±0.8	2.0±0.1	1.5±0.2
	18:0	2.3±1.9	2.3±1.9	1.9±0.2	1.6±0.4
	18:1	3.8±1.3	5.9±2.6	7.7±2.0	9.1±2.0
10	18:2	37.3±3.7	62.2±5.9	15.7±11.7	24.4±14.9
	18:3	31.9±4.5	6.7±0.7	51.3±10.9	42.1±15.5

Table 2 shows the fatty acid composition of transgenic roots. The transgenic roots resulting from infection of wild type or the fad3 mutant with *A. tumefaciens* R1000 carrying the vector (pBI121) or the plasmid pTiDES3 were grown in the presence of kanamycin (50 g/ml) for three weeks to identify the roots which had been cotransformed with one of these plasmids. The fatty acid composition of the roots was determined as previously described (Browse et al., 1986). The abbreviations used in Table 2 are as follows: 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. The values presented are the mean ± SD (n=12).

From these results it can be seen that the production of rooty tumors containing pBI121 on wild type *Arabidopsis* or the fad3 mutant had no effect on the fatty acid composition over non-pBI121 containing wild type *Arabidopsis* or fad3 mutant. By contrast, transformation of the fad3 mutant with the plasmid pTiDES3 resulted in large increases in the content of linolenic acid. In contrast to the linolenic acid content of 6.7 ± 0.7% in the fad3 mutant transformed with pBI121, the presence of pTiDES3 resulted in accumulation of 42.1% of the fatty acids as linolenic acid. The increased content of linolenic acid was accompanied by a

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decrease of corresponding magnitude in the content of linoleic acid. Thus, it is clear that the fad3 gene encodes a linoleic acid desaturase. Introduction of the fad3 gene into wild type tissues also resulted in significantly increased accumulation of linolenic acid and a corresponding decrease in linoleic acid (Table 2). Thus, it is apparent from these results that the linoleic acid content of plant tissues can be increased by high level expression of a linoleic acid desaturase. In the present embodiment, the fad3 gene was placed under transcriptional control of the constitutive high level CaMV 35S promoter carried on pBI121. The implication from these results is that expression from this promoter raised the level of expression of the fad3 gene to levels higher than are normally achieved by expression from the endogenous fad3 promoter. The results presented here indicate that the fad3 gene has significant utility in genetic modification of higher plants to elevate linolenic acid levels.

15 Example 2

Antisense expression of fad3 gene to decrease linolenic acid levels

In order to decrease the linoleic acid desaturase activity by genetic engineering methodology, the cDNA insert of pBNDES3 was cloned into plant expression cassettes in an antisense orientation. A 959bp BglII restriction fragment of pBNDES3 was used in the antisense expression vectors. The fragment is from 152 nucleotides downstream of the initiating methionine codon of the cDNA to a second BglII restriction site that is located near the C-terminus of the coding region. 189 nucleotides of the coding region are excluded from this fragment. Triple ligations were performed with the fad3 gene fragment to construct two separate plant expression cassettes.

A seed specific expression cassette was constructed by insertion of the BglII fragment of pBNDES3 in an antisense orientation behind the soybean promoter for the α' subunit of β -conglycinin (7S promoter). A

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975bp HindIII to BglII fragment containing the 7S promoter derived from pMON529 was prepared by digesting with BglII for 30min at 37 °C followed by addition of Calf Intestinal Alkaline Phosphatase (CIAP) (Boehringer Mannheim). The reaction was allowed to proceed for 20min followed by
5 purification of the linearized DNA using the GeneClean (Bio 101) purification system. The DNA was then digested with HindIII. A fragment derived from pMON999 containing the Nopaline synthase 3' region and the pUC vector backbone was prepared by digestion with BamHI and treatment with CIAP. The DNA was purified by the GeneClean procedure
10 and digested with HindIII. The fragment of pBNDES3 was prepared by digestion with BglII. The three fragments were purified by agarose gel electrophoresis and the GeneClean procedure. 50 to 200ng of the purified fragments were ligated for one hour at room temperature followed by transformation into the *E. coli* strain JM101. Resulting transformant
15 colonies were used for plasmid preparation and restriction digestion analysis. Double digestion with BglII and NcoI was used to screen for transformants containing the fad3 gene in an antisense orientation. One clone was designated as correct and named pMON13801.

A second expression cassette was constructed to allow for
20 constitutive expression of the antisense message in plants. A fragment containing the enhanced 35S promoter was prepared from pMON999 by restriction digestion with HindIII and BglII followed by treatment with CIAP as above. The correct sized fragment was obtained by agarose gel electrophoresis and the GeneClean procedure. The BglII to HindIII vector
25 fragment and the BglII fragment of pBNDES3 which were purified above were used in this construction. Ligation, transformation and screening of clones were as described above. One clone was designated as correct and named pMON13802.

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In both pMON13801 and pMON13802, the promoter, fad3 gene and the Nos 3' region can be isolated on a NotI restriction fragment. These fragments can then be inserted into a unique NotI site of the vector pMON17227 to construct glyphosate selectable plant transformation
5 vectors. The vector DNA is prepared by digestion with NotI followed by treatment with CIAP. The fad3 containing fragments are prepared by digestion with NotI, agarose gel electrophoresis and purification with GeneClean. Ligations are performed with approximately 100ng of vector and 200ng of insert DNA for 1.5 hours at room temperature. Following
10 transformation into the E. coli strain LE392, transformants were screen by restriction digestion to identify clones containing the fad3 expression cassettes. Clones in which transcription from the fad3 cassette is in the same direction as transcription from the selectable marker were designated as correct and named pMON13804 (FMV/CP4/E9, 7S/anti fad3/NOS)
15 (Figure 8) and pMON13805 (FMV/CP4/E9, E35S/anti fad3/NOS) (Figure 9).

In preparation for transforming canola cells, pMON13804 and pMON13805 were mated into Agrobacterium ABI by a triparental mating with the helper plasmid pRK2013.

20 Seeds from the plants produced by transformation were analyzed for alterations in fatty acid profile. Fatty acid methyl esters (FAMES) were prepared from seed tissue and analyzed by capillary gas chromatography (Browse et al, 1986). For initial screening of plants, six seeds were pooled together from an individual plant. The seeds were
25 crushed and FAMES extracts were made. Control plants, plants transformed with the selectable marker only (pMON17227), were also analyzed using the identical procedure. From the initial screen on pooled seed samples, several lines were identified which displayed a decreased level of linolenic acid. Lines with decreased levels of linolenic acid were

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reanalyzed by determining fatty acid profiles from individual seeds. Four to twenty individual seed were analyzed from candidate lines and from selected control plants. The results of the FAMES analysis is summarized in Figure 9.

- 5 Figure 9 shows the levels of fatty acids expressed in molar percent of twenty individual seed of the transgenic line 13804-51 as compared to control seed. Panel A discloses oleic acid, panel B discloses linoleic acid and panel C discloses linolenic acid.

- 10 The data in Figure 9 demonstrate that antisense expression of a linoleic acid desaturase has significantly altered the fatty acid profile of the resulting seed tissue. The percent of linolenic acid has been reduced to a little over 2% of the total fatty acid in the seed tissue. The percent of linoleic acid has been reduced slightly and surprisingly, the percent of oleic acid in the seed has been increased to approximately 70%. This
15 demonstrates the applicability of utilizing the fad3 gene to manipulate the fatty acid profile of crop plants.

- In order to demonstrate that the alteration in the fatty acid profile of the FAMES extracted from total seed tissue would be reflected in the seed oil fraction, triglycerides from seeds of fad3 antisense plants were
20 characterized. Total lipid extracts were made by pooling ten seeds and grinding in 2ml of methanol:chloroform:water (4:2:1). The homogenate was allowed to stand for 20min and then debris was pelleted and discarded. To the supernatant 400µl of chloroform:methanol (2:1), 640µl of chloroform and 740µl of water was added and vortexed. Phases were separated by
25 centrifugation and the chloroform phase was recovered and dried under nitrogen. Samples were resuspended in 100µl of chloroform and 10µl was applied to silica gel G thin layer chromatography plates for separation. Two identical plates were prepared with one being charred after development to allow for alignment and location of spots to be analyzed on

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the other plate. Plates were developed three times in petroleum ether:diethyl ether:acetic acid (90:10:1). One plate was sprayed with 50% sulfuric acid and heated in an oven at 90°C to allow for detection of lipids. Triglyceride fractions were identified as comigrating on the plate with purchased lipid standards (Sigma Chemical Co, cat #178-13). The charred plate was aligned with the identical plate and the triglyceride fractions were scraped from the plate. The fatty acids were transesterified to produce FAMES extracts for GC analysis by the same procedure as above. The fatty acid profiles of the triglyceride fractions are shown in Table 3 and demonstrate that this fraction have decreased linolenic acid.

TABLE 3

	<u>Transgenic line</u>	<u>Mol% 18:1</u>	<u>18:2</u>	<u>18:3</u>
15	17227-10	44	30	15.3
	17227-493	65	17	6.9
	13804-47	58	21	4.3
20	13804-50	67	20	2.8
	13804-76	59	19	5.0
	13804-117	62	21	4.0

Table 3 compares the fatty acid molar percentages of triglyceride fractions from control and transgenic lines. These above results provide clear evidence that the fad3 gene can be used to decrease the levels of linolenic acid in the storage oil of plants. The gene provides a tool for the manipulation of the fatty acid profile of seed storage oil to improve the products derived from the oil.

A surprising result of this Example 2 is the effect the antisense fad3 gene has on the oleic acid content. The precise mechanism by which

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antisense expression of a gene exerts an effect on the activity of an endogenous gene is unclear but is obviously a function of the homology of the sense and antisense gene products. Based upon the above experimental result, it would not be unreasonable to predict that the
5 portion of the fad3 gene antisense message used contained a certain degree of homology with the genes providing the activity of one or more oleate desaturases. Therefore, a further advantage of the above invention is that it is possible that expression of a linoleic acid desaturase antisense message may exert an effect on oleate desaturase activity.

10 The unexpected nature of the reduction in oleic acid desaturase activity from the antisense fad3 plants is most apparent when one compares the fatty acid profiles from the antisense plants and the fad3 mutant of *Arabidopsis*. The levels of linoleic acid in the fad3 mutant plants increased when linoleic acid desaturase activity was eliminated by
15 mutation. This indicates that the activity of the oleate desaturase was not highly effected by the loss of linoleic acid desaturase activity or by the accumulation of linoleic acid. In the fad3 mutant of *Arabidopsis* the level of linoleic acid increased when the level of linolenic acid decreased. However, a different pattern occurred in the antisense fad3 plants. In plants which
20 exhibit a decreased percent of linolenic acid there is no corresponding increase, and is often a decrease, in the percent of linoleic acid. There is an increase in the percent of oleate in the antisense fad3 plants. This would indicate that oleate desaturase activity is depressed in these plants. The effects on the fatty acid profile by the fad3 mutation and the fad3 antisense
25 expression are not equivalent, indicating that antisense expression of a linoleic acid desaturase can depress an oleate desaturase activity in plants.

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Example 3Modification of linolenic acid levels in soybean

The isolation of the *fad3* gene from *B. napus* provides a tool to those with ordinary skill in the art to isolate the corresponding gene or
5 cDNA from other plant species. There are many examples in which genes from one plant species have been used to isolate the homologous genes from another plant species. One such plant which could be improved upon by the modification of the level of linolenic acid is soybean.

Soybean oil typically contains linolenic acid at a level of 7-9% of
10 the fatty acid in the oil. This level is undesirable because it promotes instability upon heating and imparts rancidity to the finished product. The levels of linolenic acid can be lowered by the expression of the soybean *fad3* gene or cDNA in an antisense orientation in the developing seed. The following example describes one method for the isolation of a *fad3* cDNA
15 from soybean. However, similar procedures could be followed to isolate a genomic clone which could also be used to decrease the level of linoleic acid desaturase activity by antisense expression of a portion or all of the gene.

The *fad3* gene from *B. napus* is used as a probe to screen a cDNA library constructed from soybean mRNA. In order to isolate a cDNA to be
20 used in decreasing linolenic acid in seed, the optimal tissue to use for the isolation of mRNA is developing seed. There is, however, flexibility in the choice of methods and vectors which can be used in the construction and analysis of cDNA libraries (Sambrook et al, 1989). Procedures for the construction of cDNA libraries are available from manufacturers of cloning
25 materials or from laboratory handbooks such as Sambrook et.al, 1989. Once a suitable cDNA library has been constructed from soybean, all or a portion of the *fad3* cDNA from *B. napus* is labeled and used as a probe of the library. DNA fragments can be labeled for radioactive or non-radioactive screening procedures. The library is screened under suitable stringency.

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Conditions are dependent upon the degree of homology between the *fad3* gene of *B. napus* and soybean. Probe positive clones are plaque purified by standard procedures and characterized by restriction enzyme mapping and DNA sequence analysis. Clones are concluded to be soybean *fad3* based
5 upon data obtained from the sequence analysis or by expression in plants.

The entire clone or a portion thereof is placed down stream of a promoter sequence in an antisense orientation. Suitable promoters include seed specific promoters, such as the 7S (β -conglycinin) α' -subunit promoter, or less tissue specific promoters, such as the CaMV 35S
10 promoter. An appropriate 3' non-translated region is placed downstream of the antisense cDNA to allow for transcription termination and for the addition of polyadenylated nucleotides to the 3'end of the RNA sequence. This expression cassette is then combined with a selectable or scorable marker gene and soybean cells are transformed by free DNA delivery
15 (Christou, et al, 1990) or an *Agrobacterium* based method of plant transformation (Hinchee et al, 1988). Plants recovered are allowed to set seed and mature seed are used for the production of FAMES by the procedures outlined above. The FAMES extracts are analyzed by gas chromatography to identify plant lines with reduced levels of linolenic acid
20 in the seed.

Alternatives to the above methods may include but are not limited to the use of degenerate oligonucleotides as probes to screen the library. Degenerate oligonucleotide probes would be most optimally designed by choosing short segments of the *fad3* amino acid sequence where
25 the degeneracy of the genetic code is limited or by choosing sequences which appear to be highly conserved between the *fad3* gene of *B. napus* and other known linoleic acid desaturases, such as the desaturase from the cyanobacterium *Synechocystis*. The oligonucleotides could be labeled and used to probe a soybean cDNA library. Alternatively, degenerate

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oligonucleotides could be used as primers for the isolation of a portion or all of the soybean cDNA by PCR amplification.

Similar procedures could be used to isolate the homologous genes from other plant species. Another preferred plant species which could be improved upon by the modification of the level of linolenic acid is flax. Flax oil typically contains linolenic acid at a level of 45-65% of the fatty acid in the oil. This level is undesirable because it promotes instability upon heating and imparts rancidity to the finished product.

Example 4

10 Sense expression of fad3 to obtain reduced levels of linolenic acid

The cloning of the fad3 gene also provides a tool to decrease the levels of linolenic acid via the mechanism of co-suppression. The molecular mechanism of co-suppression occurs when plants are transformed with a gene that is identical or highly homologous to an allele found in the plants genome (Bird and Ray, 1991). There are several examples where expression of a chimeric gene in plants can result in a reduction of the gene product from both the chimeric gene and the endogenous gene(s). Therefore the fad3 gene product of *B. napus* may be reduced by transformation of *B. napus* with all or a portion of the fad3 cDNA which has been isolated. The resulting plant has reduced linoleic acid desaturase activity in tissues where the chimeric gene is expressed. The phenotype of reducing the linoleic acid desaturase activity is a reduction in the levels of linolenic acid. The mechanism of co-suppression could be applied to any plant species from which the fad3 gene is cloned and the plant species is transformed with fad3 in a sense orientation.

In order to reduce levels of linolenic acid by the mechanism of co-suppression, a plant transformation construct is assembled with the fad3 gene or cDNA in a sense orientation. The entire clone or a portion thereof is placed downstream of a promoter sequence in a sense orientation. Suitable

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promoters include seed specific promoters, such as the 7S (β -conglycinin) α' -subunit promoter, or less tissue specific promoters, such as the CaMV 35S promoter. An appropriate 3' non-translated region is placed downstream of the fad3 gene to allow for transcription termination and for the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. This expression cassette is then combined with a selectable marker gene and *B. napus* cells are transformed by an *Agrobacterium* based method of plant transformation. Plants recovered are allowed to set seed and mature seed are used for the production of FAMES which are analyzed by gas chromatography to identify plant lines with reduced levels of linolenic acid in the seed.

Example 5

Isolation of a chloroplast delta 15 desaturase from *Arabidopsis*

A fragment of 959bp was excised from the fad3 cDNA insert using the restriction endonuclease BglII, and labeled radioactively according to Feinberg and Vogelstein (1983). This fragment was used to probe a cDNA library from *Arabidopsis thaliana* as described above (Example 1) except that the hybridization temperature was 52° C. Several cDNA clones were positive, and one of them (pVA1) was further characterized. Its deduced amino acid sequence exhibited a strong homology with fad3 except at the N-terminus. The cDNA insert was placed under the control of the 35S promoter in the Ti vector pBI121, and the resulting construct, pBIVA12 was electroporated into *Agrobacterium* (C58 pGV3101). The bacterium was used to transform the *Arabidopsis* mutant fadD. For transformation, plants were grown at 22° C with a light intensity of 100/ μ E/cm², until bolting (approximately 2 and 1/2 weeks). The stems (1mm-10mm long) were removed and the plants were inoculated with a drop of an overnight culture of the bacterium. The same operation was repeated 7 days afterwards.

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The plants were then allowed to set seeds. The seeds were plated (2500 seeds per 150mm petri dish) on MSO plates that contained 50µg/ml kanamycin to select for plants that had integrated the construct. One transformant plant was obtained, and the fatty acids from its leaves were analyzed by gas chromatography (Table 4). The results obtained show that the pBIVA12 construct is able to reestablish the levels of linolenic and hexadecatrienoic acids in the fadD mutant at a level equal to or superior to the wild type. This demonstrates that pVA12 encodes the fadD gene.

10

TABLE 4

	fatty acid	fadD	WT	FadD pBIVA12
15	16:0	13.0	14.0	14.9
	16:1	4.9	4.3	4.2
	16:2	8.7	0.5	0.3
	16:3	3.0	13.2	9.5
20	18:1	3.3	2.3	1.2
	18:2	36.4	10.9	5.8
	18:3	30.8	54.6	63.7

Table 4 shows the complementation of the fadD mutant. Fatty acids were extracted from leaves of *Arabidopsis* according to Browse et al (1986) and were quantified (mol%) by gas chromatography. WT stands for the Columbia wild type.

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Example 6Isolation of a second chloroplast delta 15 desaturase from *Arabidopsis*

A fragment of 959 bp was excised from the cDNA insert using the restriction endonuclease BglII, and labelled radioactively according to
5 Feinberg and Vogelstein (1983). This fragment was used to probe a cDNA library from *Arabidopsis*, exactly as described above (Example 5). Among the several positive clones obtained, the cDNA pVA34 was further characterized. Its deduced amino acid sequence exhibited 71.8% and 79.5% homology with fad3 and fadD, respectively. The N-terminus resembled a
10 chloroplast transit peptide, meaning that this protein is likely to be localized to the chloroplast. The strong homology with fad3 and fadD suggests that the protein is also a delta 15 desaturase. Aside from fad3 and fadD, the only locus known to control delta 15 desaturation is the fadE locus, which controls a temperature-induced delta 15 desaturase.
15 Therefore, it is likely that the cDNA contained within the clone pVA34 corresponds to the fadE locus.

Example 7Linoleic desaturase homology to plant oleic desaturases

The linoleic desaturase genes are the first plant desaturases
20 isolated whose proteins enzymatically perform the desaturation of an unsaturated fatty acid precursor. The reaction that linoleic desaturase performs and the cofactors it uses are likely to be very similar for the oleic desaturase reaction. Given the similar reactions, similar substrates and probably similar cofactors, it is likely that the oleic desaturase genes and
25 proteins have homology to the linoleic desaturase genes and proteins. That the genes share homology is supported by the finding that antisense expression of the linoleic acid desaturase message results in higher oleic acids levels, which experimentally indicates homology between the linoleic and oleic desaturases. These factors indicate that the linoleic desaturase

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protein and nucleic acid sequences provide useful information for isolating other lipid desaturase genes, particularly oleic desaturase genes.

a. Identification of unknown cDNA sequences in databases.

5 Random cDNA sequencing generates a large number of sequenced clones but provides no information about the function of the encoded proteins. Homology to known proteins is the quickest method for identifying the protein function encoded in the sequenced cDNA. However, homology searches are informative only when a homology with a previously
10 characterized protein are found. A cDNA sequence that is not homologous to any known protein remains in the unknown function category. Thus the results functionally identifying the linoleic desaturases by sequence and by their ability to complement mutations in plant desaturase genes now provides a method for identifying the function and identity of random cDNA
15 clones by their homology to the linoleic desaturases. Additionally oleic desaturases are identified by their homology with linoleic desaturases.

A TFASTA search of the GenBank and EMBL public data bases for genes encoding proteins homologous to the protein sequence of the linoleic desaturase fad3 has identified both linoleic desaturases and a
20 second class of plant lipid desaturases likely to be oleic desaturases. In particular, sequences found in GenBank and EMBL and identified as T04093 and T12950 show significant homology to linoleic desaturases but show less homology than other linoleic desaturases. These sequences have 30% homology to fad3 and 56% similarity to fad3 linoleic desaturase
25 (TABLE 5). The full length clone of these cDNAs is obtained by standard methods and is inserted into plant gene expression and transformation vectors and transformed into fad2 Arabidopsis mutants to confirm the identity of the oleic desaturase by genetic complementation as was described in the example with linoleic desaturase.

Comparison of Fad3 and T04093 Protein Sequences

5

Percent Similarity: 52.381% Percent Identity: 30.476%

```

10 fad3 101 GHGSFSDIPLLNSVVGHILHSFILVPYHGWRISHRTHHQNHGHVENDESW 150
    |::|::||| :|.|| || |:|.|| :
T04093 1 .....LIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVF 34

15 151 VPLPEKLYKNLP.....HSTRMLRYTVPLPMLAYPIYLWYRSPGKEGSHF 195
    || ... .| .. .|.::..||.: ::::|| :. .|: .:
    35 VPKQKSAIKWYGKYLNNPLGRIMMLTVQF.VLGWPLYLAFNVSGR...PY 80

20 196 NPYSSLFAPSERKLIATSTTCWSIMLATLVYLSFLVDPVTVLKVYGVPIYI 245
    ::::| |... . . . .: :
    81 DGFACHFFPNAPIYNDRERSRYTSLMRVF*..... 110

```

b. Isolation of a oleic desaturase cDNA

25

25 The protein sequence of plant linoleic desaturases can be used
to isolate oleic desaturases. The conserved regions between the linoleic
desaturases and the DesA oleic desaturase are functionally important and
are conserved in the plant oleic desaturase proteins as well. These
conserved amino acid sequences provide a method of isolating plant oleic
30 desaturases. There are several regions of the linoleic desaturase fad3 that
are conserved in fadD, fadE and DesA. The consensus amino acid sequence
is shown in Table 6, with the amino acids identical in all four proteins shown
in capital letters. As described below, oligonucleotides designed to encode
the amino acids sequences in the conserved regions are used to identify and
35 isolate plant oleic desaturases.

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TABLE 6
Pad3 Protein Sequence and Peptide Targets

5	<p>MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFKIGDIRAAIPKHCWKSPLRSMSYVTRD v.tplttp ...spseed..erfdpgapppf.laDiraaIPKhCwvKnpwksmsyVvrd <u>DiraaIPKhCwvK</u> (1a) DiraaIP (1b) aiPKhC (1c) KhCwvK</p>
10	<p>IFAVAALAMAAVYFDSWFLWPLYWVAQGLFWAIFVLGHDCGHGFSFSDIPLLNSVVGHIL va.vfalaa.aayfnnW.lwPlyW.aqGTmfwalFVLGHDCGHGFSsndp.lNsvvGH.l <u>WflwPlyWvaqGT</u> <u>FVLGHDCGHGFS</u> (2a) WflwPlyW (3a) FVLGHDC (2b) WflwP (3b) VLGHDC (2c) wPlyW (3c) GHDCGH (2d) WvaqGT (3d) CGHGSF</p>
20	<p>HSFILVPYHGWRISHRTHHQNHGHVENDSWVPLPEKLYKNLPHSTRMLRYTVPLPMLAY hssilvPyHgWRisHrthHHqnhghvEnDesWhPl.ekiyknlpk.trmftrftlplpmlay <u>PyHgWRisHrthHH</u> <u>EnDesWvP</u> (4a) PyHgW (5a) EnDesW (4b) HgWRisH (5b) DesWvP (4c) WRisHrthHH (4d) WRisH (4e) HrthHH</p>
30	<p>PIYLWYRSPGKEGSHFNPYSSLFAPSERKLIATSTTCWSIMLAT.LVYLSFLVDPVTVLK pfylw.rspgk.gShyhpds.lF.pkerkdvltStacwtamaAl.lvcLnft.gpiqmlk VYGVPIIIFVMWLDVAVTYLHHHGHGDEKLPWYRGKEWSYLRGGL.TTIDRDYG.IFNNIH lygiPywifvmWldfvTylHHHghedklpwyrgkeWSylrggL.tTldrDYg.winnih <u>WldavTylHH</u> <u>WSylrggL.tTldrDY</u> (6a) WldavT (7a) WSylrggL (6b) TylHH (7b) L tTldrD (7c) TldrDY</p>

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HDIGTHVIHHLFPQIPHYHLVDATRAAKHVLGRYYREPKTSGAIPHLVESLVASIK
 HDIgtHviHHLfpqIPhYhLveAteaaKpvlGkyrEpk.sgplplhLlesl.ksik
HDIgtHviHHLfpqIPhY

- 5 (8a) HDIgtH
 (8b) HviHHL
 (8c) HHLfpqI
 (8d) HLfpqIP
 (8e) LfpqIPhY

10

KDHYVSDTGDIVFYETDPDLVYASDKSKIN*
 .dhyvsdtGdvvyYeadp.lyg..s*

15 c. Isolation of the fadC (fad6) Gene from *Arabidopsis thaliana*

The fadC gene (also referred to as fad6) encodes a chloroplastic omega-6 desaturase.

- The deduced amino acid sequences of the fad3 gene from *Brassica napus* and the fadD and fadE genes from *Arabidopsis thaliana*
 20 were compared with the DesA gene from *Synechocystis* (*Nature*, 347:200, 1990). The sequence GHDCGH was determined to represent the most highly conserved region of these proteins. Consequently, a degenerate oligomer was designed that contains all the possible condons for the sequence GHDCGH. This oligomer has the following sequence:
 25 GGNCAYGAYTGYGGNCA.

An *Arabidopsis thaliana* cDNA phage library obtained from the laboratory of Dr. Ron Davis (*PNAS*, 88: 1731-1735) was used to screen for desaturase genes. This library was made using material from all above ground plant parts.

- 30 Approximately 120,000 phage from the library were plated onto three plates and hybondN+ was then used to prepare three filters from each plate (*Molecular Cloning - A Laboratory Manual*, 2nd Edition. Eds. J. Sambrook, E. F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory

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Press, Cold Spring Harbor, New York 1989, hereafter "Sambrook"). Two filters from each plate were probed using the degenerate consensus oligomer which had been end-labelled with (32)P using T4 polynucleotide kinase. The hybridizations were performed in a solution that contained high
5 amounts of tetramethylammonium chloride in order to minimize differences in the melting temperatures of the oligomers that together comprise the degenerate consensus oligomer. The hybridization solution had the following composition: 3 M tetramethylammonium chloride, 10 mM sodium phosphate pH 6.8, 1.25 mM EDTA, 0.5% SDS, 0.5% milk. Hybridization
10 was carried out overnight at a temperature of 44°C. Filters were then washed four times, 20 minutes each time, with 6 x SSC + 0.15% SDS at room temperature. Filters were then washed one time, for 30 minutes, with 4 x SSC + 0.1% SDS at room temperature. The filters were then exposed to film for two days.

15 The third set of filters that were made from each phage-containing plate were probed using DNA sequences from the three *Arabidopsis* desaturase genes that had already been identified: fad3, fadD and fadE. The fad3, fadD and fadE genes were labelled with (32)P and hybridized to the third set of phage filters in the following hybridization
20 solution: 0.2 M NaCl, 20mM sodium phosphate pH 7.7, 2mM EDTA, 1% SDS, 0.5% milk, 10% dextran sulfate, 0.1% sodium pyrophosphate. Hybridization was carried out overnight at 65°C. Filters were washed four times, 30 minutes per time, in 2 x SSC + 0.15% SD at room temperature and then for 45 minutes with 1 x SSC + 0.1% SDS at 65° C. The filters
25 were then exposed to film for approximately two hours.

The two sets of filters that were probed with the degenerate consensus oligomer showed about 60 positive phage per plate (or about 180 total positive phage). Results from the third set of filters that were probed with the fad3, fadD and fadE genes indicated that only a small percentage

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of the phage that hybridized to the consensus of oligomer contained the fad3, fadD or fadE genes.

Seventy-six of the phage that hybridized to the consensus oligomer, but not to the fad3, fadD or fadE genes, were plaque purified. The
5 purified phage were then spotted onto bacteria growing on solid media on plates and allowed to form plaques. Several duplicate filters were then made of these plates (Sambrook). One of these filters was probed with the consensus oligomer, as described above. A second filter was probed with a mixture of the *Arabidopsis thaliana* fad3, fadD and fadE genes, as
10 described above.

In order to determine which of the 76 phage contained the same cDNA inserts as which other phage, some of the filters were probed with cDNA inserts from some of the phage. In order to perform this experiment, the cDNA inserts from most of the phage were isolated by
15 using oligomers that bound to DNA flanking the cDNA cloning site in the phage vector to isolate the cDNA sequences using the polymerase chain reaction (PCR). These cDNA sequences were labelled with (32)P (random hexamer labelling) and hybridized to the filters using the following hybridization solution: 30% formamide, 0.2M NaCl, 20mM sodium
20 phosphate pH 7.7, 2mM EDTA, 1% SDS, 0.5% milk, 0.1% sodium pyrophosphate. The hybridizations were carried out for 14 hours at 65°C. The filters were washed four times 15 minutes per wash, with 2 x SSC + 0.15% SDS at room temperature and were then exposed to film.

The combination of the high formamide concentration in the
25 hybridization solution and the high hybridization temperature meant that only DNA sequences that were virtually identical would hybridize, allowing us to distinguish between nearly identical sequences. Several rounds of hybridizations using cDNA inserts from different phage were carried out until it had been determined which phage contained the same, or at least

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extremely similar, cDNA inserts. On the basis of these experiments, we determined that all of the 76 phage contained one of four cDNA inserts. Sequence data was obtained from each of these four cDNAs. None of these cDNAs was found to be homologous to known desaturase genes, and so we
5 feel that none of these four cDNAs is likely to encode a desaturase.

Since the number of phage that hybridized to the consensus oligomer was quite high (about 180 phage hybridized in the initial screen described above), we were not able to analyze all of the positive phage in the initial experiments. So, an attempt was made to identify phage that
10 hybridized to the consensus oligomer but that did not contain the *fad3*, *fadD* or *fadE* genes or one of the four non-desaturase encoding clones that were identified in the first screen. In order to do this, between 500,000 and 1,000,000 phage from the library described above were plated onto 10 plates. Three filters were made from each plate (Sambrook). Two of these
15 three sets of filters were then hybridized with (32) P labelled consensus oligomer as described above except that hybridization was carried out at 42°C instead of at 44°C. The third set of filters were hybridized with (32)P labelled DNA from the *Arabidopsis* *fad3*, *fadD* and *fadE* genes together with DNA from each of the four cDNA's identified in the first round of screening
20 as hybridizing to the consensus oligomer but not encoding desaturases. This third set of filters were hybridized in: 30% formamide, 0.2 M NaCl, 20mM sodium phosphate pH 7.7, 2mM EDTA, 1% SDA, 0.5% milk, 0.1% sodium pyrophosphate at 65°C. All three sets of filters were hybridized for 12 hours and then washed several times with 2 x SSC + 0.15% SDS at
25 room temperature. The filters were then exposed to film.

Approximately 200 phage from each plate hybridized to the consensus oligomer. 50-60% of these phage also hybridized to *fad3*, *fadD*, *fadE* or to one of the four clones identified in the first screen. About 58 phage that hybridized to the consensus oligomer, but not to *fad3*, *fadD*,

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fadE or one of the four previously identified clones, were plaque purified. The purified phage were then spotted onto a bacterial lawn growing on solid media on a petri plate and the phage were allowed to form plaques. Several filters were prepared from these plates and hybridized with (32)P labelled
5 cDNA inserts from various of the newly purified phage, as described above. In this manner, all of the phage identified in this second round of screening were found to contain one of eight different cDNA inserts.

Sequence data was obtained from each of the eight cDNA's. One of the cDNA's, which was contained within only one of the phage, was
10 found to have some sequence similarity of a known desaturase gene from cyanobacteria, the DesA gene. Further sequence information was obtained for this clone. This additional sequence showed very significant sequence similarity to the DesA gene, confirming that the clone contained a desaturase gene. The remainder of the cDNA contained within the clone
15 was sequenced and compared with the sequences of other known desaturases. The new desaturase was 53.0% identical to DesA at the nucleotide level and 43.9%, 45.6% and 47.0% identical to *B. napus* fad3, *Arabidopsis* fadD and *Arabidopsis* fadE, respectively. As the gene contained within the clone was significantly more similar in sequence to the
20 DesA gene (which is a delta-12 desaturase) than to fad3, fadD or fadE (which are omega-3 desaturases), the new desaturase was expected to be a delta-12 (= omega-6) desaturase.

The additional sequence data also indicated that this new desaturase gene contains a region that has only a one base pair mismatch
25 to the desaturase consensus sequence described above. This mismatch means that the new desaturase has the sequence GHDCAH instead of GHDCGH.

A clone containing a full length cDNA for this gene was isolated and completely sequenced. This full length cDNA was sub-cloned

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into the plant transformation vector pBII121 such that the gene is transcribed under the control of the 35S promoter. This construct was then used to complement the phenotype of a fadC mutant (*Plant Phys.* 90: 522-529, 1989) of *Arabidopsis thaliana*, indicating that the gene encodes a chloroplastic omega-6 desaturase.

d. Proposed isolation of fad2

The most highly conserved peptide regions in the linoleic desaturases and the DesA desaturase were chosen as regions likely to be conserved in oleic desaturases. These 8 conserved regions are shown in TABLE 6. These regions were chosen on the following basis: These regions have areas highly conserved between the 3 linoleic desaturases and DesA, with at least 4 identical amino acids over a 10 amino acid span. Once a region was identified as conserved, the fad3 linoleic desaturase sequence was used as the amino acid sequence for the source of homology to identify oleic desaturases. This is because both fad3 and the non-plastid oleic desaturases are thought to be localized to the endoplasmic reticulum and are most likely to contain similar amino acid sequences.

Several peptide endpoints in each conserved area were chosen as the basis to subsequently design oligonucleotide probes for identifying the oleic desaturase gene. The peptide endpoints were chosen to be between 5 and 9 amino acids in length. The peptide end points were chosen to end on the conserved (identical) amino acids, and most often to begin on conserved amino acids. The rationale is that within the larger conserved area, some amino acid portions are more highly conserved than others, that 15 to 27 (5 to 9 amino acids) nucleotides is a good primer size for PCR, and that for PCR it is important that the 3' end of the primer matches the target, with the conserved (identical) amino acids the most likely to be present in the oleic desaturases. These 28 "oleic desaturase" peptide targets (Table 6) are the basis oligonucleotides that are designed for

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hybridizing to the oleic desaturase cDNA sequences to identify and isolate the oleic desaturase cDNA clone.

- Several possible methods for designing oligonucleotides and isolating the genes encoding the target peptide regions are known. For a
- 5 discussion of designing degenerate oligonucleotides see *PCR Protocols - A Guide to Methods and Applications*, Eds M. A. Innis, D. H. Gelfand, J J Sninsky and T. J. White, Academic Press, San Diego, California, 1990; and Sanxxxxx The two most common screening methods using the oligonucleotides are screening cDNA libraries and PCR amplification of
- 10 specific cDNAs. Gene probes from *fad3*, *fadD* and *fadE* are used under stringent hybridization conditions to identify these cDNAs and discard them in the screen for oleic desaturase cDNA clones. The method for using degenerate oligonucleotides to screen a cDNA library has been described in the example above demonstrating the isolation of the *fadC* oleic desaturase
- 15 gene. An immature plant seed active in oil biosynthesis, generally 2 to 5 weeks after pollination, preferably about 3 to 4 weeks after pollination, of a plant such as *Arabidopsis* or canola is used as the source of mRNA for making cDNA. First strand cDNA is made from the isolated mRNA and hybridized under stringent conditions in solution to an excess of biotinylated
- 20 *fad3*, *fadD* and *fadE* cloned cDNAs. The hybrids and biotinylated nucleic acids are removed with streptavidin and a second round of subtraction is done to remove any remaining *fad3*, *fadD* and *fadE* sequences. The cDNA remaining in solution is used for PCR reactions. (For 5' RACE, see below, a polyA tail is added to the first strand cDNA 3' end).
- 25 A method that can readily evaluate a number of degenerate oligonucleotides probes is degenerate PCR (See chapters by Compton and by Lee and Caskey in *PCR Protocols*, cited above). In this method a degenerate set of oligonucleotides encompassing all the possible codon choices for the target peptide is synthesized (such degenerate

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targets (Table 6) are the basis oligonucleotides that are designed for hybridizing to the oleic desaturase cDNA sequences to identify and isolate the oleic desaturase cDNA clone.

Several possible methods for designing oligonucleotides and isolating the genes encoding the target peptide regions are known. For a discussion of designing degenerate oligonucleotides see *PCR Protocols - A Guide to Methods and Applications*, Eds M. A. Innis, D. H. Gelfand, J J Sninsky and T. J. White, Academic Press, San Diego, California, 1990; and Sambrook. The two most common screening methods using the oligonucleotides are screening cDNA libraries and PCR amplification of specific cDNAs. Gene probes from fad3, fadD and fadE are used under stringent hybridization conditions to identify these cDNAs and discard them in the screen for oleic desaturase cDNA clones. The method for using degenerate oligonucleotides to screen a cDNA library has been described in the example above demonstrating the isolation of the fadC oleic desaturase gene. An immature plant seed active in oil biosynthesis, generally 1 to 5 weeks after pollination, preferably about 2 to 4 weeks after pollination, of a plant such as Arabidopsis or canola is used as the source of mRNA for making cDNA. First strand cDNA is made from the isolated mRNA and hybridized under stringent conditions in solution to an excess of biotinylated fad3, fadD and fadE cloned cDNAs. The hybrids and biotinylated nucleic acids are removed with streptavidin and a second round of subtraction is done to remove any remaining fad3, fadD and fadE sequences. The cDNA remaining in solution is used for PCR reactions. (For 5' RACE, see below, a polyA tail is added to the first strand cDNA 3' end).

A method that can readily evaluate a number of degenerate oligonucleotides probes is degenerate PCR (See chapters by Compton and by Lee and Caskey in *PCR Protocols*, cited above). In this method a degenerate set of oligonucleotides encompassing all the possible codon

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TABLE 7
Peptide Targets for Fad2 Cloning

	Peptide sequence	Oligo sequence 5' - 3'
5		
	1a DIRAAIP	GAYATHMGNCGNCGNATHCC
	1b AIPKHC	GCNATHCCNAARCAITG
	1c KHCWVK	AARCAITGYTGGGTNAA
	2a WFLWPLYW	TGGTTYTNTGGCCNYTNTAITGG
10	2b WFLWP	TGGTTYTNTGGCCN
	2c WPLYW	TGGCCNYTNTAITGG
	2d WVAQGT	TGGGTNGCNCARGGNAC
	3a FVLGHD	TTYGTNYTNGGNCAITGA
	3b VLGHD	GTNYTNGGNCAITGAYTG
15	3c GHDCGH	GGNCAITGAYTGYGGNCA
	3d CGHGSF	TGYGGNCAITGGNWSNTT
	4a PYHGW	CCNTAITCAITGGNTGG
	4b HGWRISH	CAITGGNTGGMGNATHWSNCA
	4c-1 WRISHRTHH	TGGMGNATHTCNCAITMGNACNCAITCA*
20	4c-2	TGGMGNATHAGYCAITMGNACNCAITCA*
	4d WRISH	TGGMGNATHWSNCAIT
	4e HRTHH	CAITMGNACNCAITCAIT
	5a ENDESW	GARAAYGAYGARWSNTGG
	5b DESWVP	GAYGARWSNTGGGTNCC
25		
	6a WLDAT	NGTNACNGCRTCNCARCCA
	6b TYLHH	RTGRTGNARRTANGT
	7a-1 WSYLRGGL	ARNCCNCCNCKNARRTARCTCCA*
	7a-2	ARNCCNCCNCKNARRTANGACCA*
30	7b LTTIDRD	RTCNCCKRTCDAITNGTNGTNA
	7c TIDRDY	RTARTCNCKRTCDAITNGT
	8a HDIGTH	RTGNGTNCCDAITRTCRTG
	8b HVIHHL	NARRTGRTGDATNACRTG
	8c HHLFPQI	DATYTGNGGGAANARRTGRTG
35	8d HLPQIP	GGDATYTGNGGGAANARRTG
	8e LFPQIPHY	RTARTGNGGDATYTGNGGGAANA

40 * synthesize 4c and 7a in two pools each to limit the degeneracy

Oligos for 6a - 8e are the complement of the coding sequence

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TABLE 8
Table of Oligomers for PCR RACE of fad2

	Peptide #	Oligo Length	Fold Degeneracy	Similarity with L26296	Similarity in Last 10 n.t.
5	1a	20	384	75 %	80 %
	1b	17	192	88	80
	1c	17	32	65	80
10	2a	24	64	79	100
	2b	15	48	73	80
	2c	15	48	100	100
	2d	17	128	76	90
15	3a	17	384	76	70
	3b	17	384	82	80
	3c	17	128	88	90
	3d	17	384	82	70
20	4a	15	64	80	70
	4b	20	192	75	90
	4c	26	96*	81	80
	4d	15	216	87	90
25	4e	15	192	87	80
	5a	18	96	72	80
	5b	17	96	76	80
30	6a	18	256	78	80
	6b	15	192	93	100
	7a	23	256*	78	60
	7b	20	384	90	80
35	7c	18	192	94	90
	8a	18	384	72	70
	8b	18	192	89	80
	8c	21	384	81	100
40	8d	20	192	80	90
	8e	23	192	83	70

* done in two oligo pools

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Table 7 shows the 28 peptide targets from the eight conserved regions and the 30 degenerate oligonucleotides derived from the peptide sequences. The degeneracy was kept to less than 516 fold, for those instances where more degeneracy occurred, by the use of deoxyinosine (Sambrook et al.) and by not including the last nucleotide in the last codon, and in two cases by the use of two subpools. Table 8 shows the amount of degeneracy for each designed oligonucleotide sequence and the amount of homology of the oligonucleotides to the *Arabidopsis* oleic desaturase fad2 (Accession No. L26296). Also shown in Table 8 is the percent homology in the last 10 nucleotides on the 3' end of each primer, since this region is most important for annealing and elongation under PCR conditions. It is expected that both 10 of 10 and 9 of 10 homology matches, and probably 8 of 10 homology matches in the 3' primer regions will serve as efficient PCR primers. Note that for oligonucleotide sets 1a through 5b (for 3' RACE) the strand direction is the same as the mRNA while for oligonucleotide sets 6a through 8e (for 5' RACE) the direction is opposite of the mRNA. Four oligonucleotides have a 10 of 10 match in the 3' position, 6 oligonucleotides match 9 of 10 in the 3' position and 12 match in 8 of 10 nucleotides in the 3' position. Oligonucleotides corresponding to peptides 2a, 2c, 2d, 3c, 4b, 4d, 6b, 7c, 8c, and 8d show 90% or greater homology in their last 10 nucleotides and anneal to the oleic desaturase gene and serve as primers to this gene. This demonstrates the validity of using the conserved regions of the plant linoleic desaturases and DesA to identify and isolate plant oleic desaturases.

The first round of PCR products are subjected to two rounds of subtraction using biotinylated fad3, fadD and fadE cloned cDNA to remove any hybridizing fad3, fadD and fadE sequences with strepavidin. This subtracted DNA is greatly enriched for fad2 sequences and depleted of fad3, fadD and fadE sequences. These 30 samples are run on agarose gels,

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blotted and hybridized with pools of probe from the 30 samples. Pools of 5 of each of the 30 PCR samples are labeled with random primers and hybridized to the blots of the 30 samples, for a total of 6 blots hybridized with 6 pools of 5 probes. Additionally, a pool of fad3, fadD and fadE probe is
5 hybridized to a duplicate blot. Bands that do not hybridize strongly to fad3, fadD and fadE but do cross hybridize to probe made from a different sample are strong candidates for fad2 as fad2 is likely to be the only DNA amplified in two or more independent PCR reactions. Positively hybridizing lanes identify samples to amplify by PCR using the same primers as in the initial
10 reaction for 5 to 10 cycles and the PCR products are cloned into plasmid vectors. The same probe that recognized the sample on the blot is used to screen the library and identify the hybridizing clone. Positive clones are sequenced and identified as fad2 clones by their homology but non-identity with fad3, and further characterized as described below.

15 In the event that fad2 sequences are not sufficiently enriched in one round of PCR to be identified, a second round of PCR is performed. If the lack of detection is due to insufficient amplification of fad2, then another round of PCR using the same primers on the subtracted PCR first round samples and the same simple screen as described above will identify
20 fad2. If there are too many competing non-specific reactions then a second round of PCR using a different primer combination will remove non-specific amplifications and enrich for fad2. To further enrich for fad2 sequences each of the initial 30 PCR samples (one for each oligonucleotide in Table 7) after subtraction as described above, is subjected to a second round of PCR
25 reactions using a different primer combination than the first reaction. One of the primers would be the same degenerate oligonucleotide primer as in the first PCR reaction. The second primer would now be from one of the 30 primers in Table 7 from the opposite class, ie, primers from 1a to 5b form matched sets with primers from 6a to 8e (primers 1a to 5b are in the sense

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direction while primers 6a to 8e are in the antisense direction). For example, if oligonucleotide 1a was used initially, it is used again as one of the two primers and the second primer is each of the 6a to 8e oligonucleotides for a total of 11 separate PCR reactions. In total the 30
5 initial reactions result in 418 second cycle PCR reactions, a number easily handled by PCR technology. Essentially this second PCR cycle accomplishes a "nested" or sequential PCR reaction step after removing all the linoleic desaturases by the subtraction step. This increases the amplification as well as the specificity. Identification of samples containing
10 fad2 are performed similarly as described above, with the 418 samples dot blotted onto 22 filters and probed with 21 pools of 20 samples and with a pool of fad3, fadD and fadE. Again, any sample that cross hybridizes with an independent probe sample and does not hybridize to fad3, fadD and fadE is a candidate for containing fad2 in the sample. If fad3, fadD and fadE
15 hybridization is still present, another biotinylation/stepavidin subtraction should remove it. Positively hybridizing samples are run on gels, the band identified by hybridization and isolated for cloning. This second set of PCR reactions produces PCR products of a predictable size since both primers are within the coding region where little variation in size is expected. Thus
20 the presence of a band of the expected size on a gel is diagnostic of fad2, particularly if hybridization of a blot of such a gel with a fad3, fadD and fadE probe indicates the band is not due to fad3, fadD and fadE contamination. After cloning the inserts in *E. coli*, the resulting plasmids containing the insert are identified by hybridization. They are sequenced
25 and identified as oleic desaturases by their homology but non-identity with the linoleic desaturases, as in the examples described previously. The full length clone of these cDNAs is obtained by standard methods and inserted into plant gene expression and transformation vectors and transformed into *Arabidopsis* fad2 mutants to confirm the identity of the oleic

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desaturase by genetic complementation as was described in the example with linoleic desaturase.

Thus in this approach to isolating the plant oleic desaturases, the total number of peptide regions is 8, comprised of 28 smaller peptide targets. This leads to set of 30 degenerate oligonucleotides, that are used in the PCR amplification and screening of the PCR products. Subtraction of interfering fad3, fadD and fadE sequences is used at several points. If necessary a second round of PCR reactions with paired internal primers gives extra amplification and specificity. This approach identifies the plant oleic desaturases, and the sequence of the isolated clones should confirm their identity by their homology to the plant linoleic desaturases as described. Thus a defined approach to isolating the plant oleic desaturases from the information about linoleic desaturases is presented here. The example given here is for Arabidopsis or canola oleic desaturases, but the approach is not limited to those plants as the oleic desaturases are probably highly conserved in most plants. Thus once one plant oleic desaturase is isolated, the sequence information is used to isolate the genes from other plant species by direct hybridization or by an approach similar to the one described here.

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The following documents, which are cited in the above application, are hereby incorporated by reference.

5 Ammirato, P.V., et al. Handbook of Plant Cell Culture - Crop Species. Macmillan Publ. Co. (1984).

Bartlett, S.G., A.R. Grossman, & N.H. Chua. (1982) In Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, New York, pp 1081-1091.

10

Beachy, R. N., Chen, Z. L., Horsch, R. B., Rogers, S. G., Hoffmann, N. J., and Fraley, R. T. (1985) EMBO J, 4:3047-3053.

15 Benfey, P., Ren, L., and Chua, N.H. (1989) EMBO J, Vol.5, no.8, pp 2195-2202.

Bevan, M. (1984) Nucleic Acids Res. 12 (22): 8711-8721.

Bird, C. R., and Ray, J. A. (1991) Biotech. Gen. Engin. Rev. 9:207-227.

20

Bray, E. A., Naito, S., Pan, N. S., Anderson, E., Dube, P., and Beachy, R. N. (1987) Planta 172:364-370.

Browse J., McCourt P., Somerville C. R. (1985) Science 227:763-65.

25

Browse, J., P.J. McCourt, C.R. Somerville. (1986) Anal. Biochem. 152:141-146.

SUBSTITUTE SHEET (RULE 26)

-65-

- Browse, J., P. McCourt, C.R. Somerville. (1986b) *Plant Physiol.* 81:859-864 .
- Browse, J. A., Slack, C. R. (1981.) *FEBS Lett.* 131:111-14
- 5 Browse, J., Somerville, C.R. (1991) *Ann. Rev. Plant Physiol. Mol. Biol.* 42:467-506 .
- Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S., Meyerowitz, E.M.
10 (1988) *Proc Natl Acad Sci USA* 85:6856-6860
- Christou, P., McCabe, D. E., Martinell, B. J., and Swain, W. F. (1990)
Trends Biotechnol 8:145-151.
- 15 Church, G.M., and Gilbert, W. (1984) *Genomic sequencing. Proc Natl Acad Sci USA* 81:1991-1995
- Doyle, J. J., Schuler, M. A., Godette, W. D., Zenger, V., and Beachy, R. N.
(1986) *J Biol Chem* 261:9228-9238.
- 20 Farmer, E. E., and Ryan, C. A. (1992) *Plant Cell* 4:129-134.
- Fromm, M., (1990) *UCLA Symposium on Molecular Strategies for Crop Improvement*, April 16-22, 1990. Keystone, CO.
- 25 Grill, E., and C.R. Somerville (1991) *Molec. Gen. Genet.*, 226:484-490.
- Harwood, J. L. (1988) *Annu Rev Plant Physiol.* 39:101-38

SUBSTITUTE SHEET (RULE 26)

-66-

- Herrera-Estrella, L., et al. (1983) Nature 303:209
- Hinchee, M.A.W., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S. J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T., Horsch, R.B.
5 (1988) Bio/Technology 6:915-922.
- Horsch, R.B. and H. Klee. (1986) Proc. Natl. Acad. Sci. U.S.A.
83:4428-32.
- 10 Hugly, S., Kunst, L., and Somerville, C. R. (1991) J. Hered. 82:484-488.
- Hugly, S., and Somerville, C. R. (1992) Plant Physiol. 99:197-202.
- Katagiri, F., E. Lam and N. Chua. (1989) Nature 340:727-730.
- 15 Kearns, E.V., S. Hugly, C.R. Somerville (1991) Arch. Biochem. Biophys.,
284:431-436.
- Klee, H.J., et al. (1985) Bio/Technology 3:637-42.
- 20 Knutson, D. S., Thompson, G. A., Radke, S. E., Johnson, W. B., Knauf, V. C.,
and Kridl, J. C. (1992) Proc. Natl. Acad. Sci. USA 89:2624-2628.
- Lemieux, B. M. Miquel, C.R. Somerville, J. (1990) Theor. Appl. Genet.,
25 80:234-240.
- Lipman, D. J., and Pearson, W. R. (1985) Science 227:1435-1441.
- McKeon T. A. , Stumpf, P. K. (1982) J. Biol. Chem. 257:12141-

SUBSTITUTE SHEET (RULE 26)

-67-

47

McCourt, P., Kunst, L., Browse, J., Somerville, C. R. (1987) Plant Physiol. 84:353-361.

5

McSheffrey, S.A., McHughen A. and Devine, M.D., (1992) Theor. Appl. Genet., 84:480-486.

Meinke, D. W., Chen, J., and Beachy, R. N. (1981) Planta 153:130-139.

10

Mersereau, M., Pazour, J., and Das, A. (1990) Gene 90:149-151.

Rogers, S., et al. (1987) In 153 Methods in Enzymology. Edited by H. Weissbach and A. Weissbach. 253: Academic Press.

15

Samac, D.A., C.M. Hironaka, P.E. Yallaly and D.M. Shah (1990) Plant Physiol. 93:907-914.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.

20

Shanklin, J., C.R. Somerville. (1991) Proc. Natl. Acad. Sci. USA 88:2510-2514.

25 Schilperoot et al, EPO publication 120,516

Schmidhauser, T.J. and D.R. Helinski. (1985) J. Bacteriol. 164: 155.

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Schmidt, H., Heinz, E. (1990a) Plant Physiol. 94:214-20

Schmidt, H., Heinz, E. (1990b) Proc. Natl. Acad. Sci. USA. 87:9477-9480.

- 5 Sherman, F., Fink, J., and Hicks, J. B. (1986) Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shimamoto, K. et al. (1989) Nature 338:274-276.

10

Steponkus, P. L., Lynch, D. V., and Vemura, M. (1990) Phil. Trans. Roy. Soc. Lond. B. 326:571-583.

Sukumaran, N.P. and Weiser, C.J. (1972) HortScience 7:467-468.

15

Vasil, V., F. Redway and I. Vasil. (1990) Bio/Technology 8:429-434.

Wada, H., Gombos, Z., Murata, N. (1990) Nature 347:200-203.

20

Vollrath, D., Davis, R.W. (1987) Nucleic Acids Res 15:7865-7876.

Ward, E.R., Jen, G.C. (1990) Plant Mol Biol 14:561-568.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Monsanto Company
- (B) STREET: 800 North Lindbergh Boulevard
- (C) CITY: St. Louis
- (D) STATE: Missouri
- (E) COUNTRY: United States of America
- (F) POSTAL CODE (ZIP): 63167
- (G) TELEPHONE: (314)694-3131
- (H) TELEFAX: (314)694-5435

(ii) TITLE OF INVENTION: Altered Linolenic and Linoleic Acid Content
in Plants

(iii) NUMBER OF SEQUENCES: 72

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/156551
- (B) FILING DATE: 22-NOV-1993

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/014431
- (B) FILING DATE: 05-FEB-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1353 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 87..1238

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATCCATCAA ACCTTTATTC ACCACATTTC ACTGAAAGGC CACACATCTA GAGAGAGAAA 60
CTTCGTCCAA ATCTCTCTCT CCAGCG ATG GTT GTT GCT ATG GAC CAG CGC AGC 113

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Met Val Val Ala Met Asp Gln Arg Ser
1 5

AAT GTT AAC GGA GAT TCC GGT GCC CGG AAG GAA GAA GGG TTT GAT CCA	161
Asn Val Asn Gly Asp Ser Gly Ala Arg Lys Glu Glu Gly Phe Asp Pro	
10 15 20 25	
AGC GCA CAA CCA CCG TTT AAG ATC GGA GAT ATA AGG GCG GCG ATT CCT	209
Ser Ala Gln Pro Pro Phe Lys Ile Gly Asp Ile Arg Ala Ala Ile Pro	
30 35 40	
AAG CAT TGC TGG GTG AAG AGT CCT TTG AGA TCT ATG AGC TAC GTC ACC	257
Lys His Cys Trp Val Lys Ser Pro Leu Arg Ser Met Ser Tyr Val Thr	
45 50 55	
AGA GAC ATT TTC GCC GTC GCG GCT CTG GCC ATG GCC GCC GTG TAT TTT	305
Arg Asp Ile Phe Ala Val Ala Ala Leu Ala Met Ala Ala Val Tyr Phe	
60 65 70	
GAT AGC TGG TTC CTC TGG CCA CTC TAC TGG GTT GCC CAA GGA ACC CTT	353
Asp Ser Trp Phe Leu Trp Pro Leu Tyr Trp Val Ala Gln Gly Thr Leu	
75 80 85	
TTC TGG GCC ATC TTC GTT CTT GGC CAC GAC TGT GGA CAT GGG AGT TTC	401
Phe Trp Ala Ile Phe Val Leu Gly His Asp Cys Gly His Gly Ser Phe	
90 95 100 105	
TCA GAC ATT CCT CTG CTG AAC AGT GTG GTT GGT CAC ATT CTT CAT TCA	449
Ser Asp Ile Pro Leu Leu Asn Ser Val Val Gly His Ile Leu His Ser	
110 115 120	
TTC ATC CTC GTT CCT TAC CAT GGT TGG AGA ATA AGC CAT CGG ACA CAC	497
Phe Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His Arg Thr His	
125 130 135	
CAC CAG AAC CAT GGC CAT GTT GAA AAC GAC GAG TCT TGG GTT CCG TTG	545
His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp Val Pro Leu	
140 145 150	
CCA GAA AAG TTG TAC AAG AAC TTG CCC CAT AGT ACT CGG ATG CTC AGA	593
Pro Glu Lys Leu Tyr Lys Asn Leu Pro His Ser Thr Arg Met Leu Arg	
155 160 165	
TAC ACT GTC CCT CTG CCC ATG CTC GCT TAC CCG ATC TAT CTG TGG TAC	641
Tyr Thr Val Pro Leu Pro Met Leu Ala Tyr Pro Ile Tyr Leu Trp Tyr	
170 175 180 185	
AGA AGT CCT GGA AAA GAA GGG TCA CAT TTT AAC CCA TAC AGT AGT TTA	689
Arg Ser Pro Gly Lys Glu Gly Ser His Phe Asn Pro Tyr Ser Ser Leu	
190 195 200	
TTT GCT CCA AGC GAG AGG AAG CTT ATT GCA ACT TCA ACT ACT TGC TGG	737
Phe Ala Pro Ser Glu Arg Lys Leu Ile Ala Thr Ser Thr Thr Cys Trp	
205 210 215	
TCC ATA ATG TTG GCC ACT CTT GTT TAT CTA TCG TTC CTC GTT GAT CCA	785

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Ser Ile Met Leu Ala Thr Leu Val Tyr Leu Ser Phe Leu Val Asp Pro	
220 225 230	
GTC ACA GTT CTC AAA GTC TAT GGC GTT CCT TAC ATT ATC TTT GTG ATG	833
Val Thr Val Leu Lys Val Tyr Gly Val Pro Tyr Ile Ile Phe Val Met	
235 240 245	
TGG TTG GAC GCT GTC ACG TAC TTG CAT CAT CAT GGT CAC GAT GAG AAG	881
Trp Leu Asp Ala Val Thr Tyr Leu His His His Gly His Asp Glu Lys	
250 255 260 265	
TTG CCT TGG TAC AGA GGC AAG GAA TGG AGT TAT TTA CGT GGA GGA TTA	929
Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu	
270 275 280	
ACA ACT ATT GAT AGA GAT TAC GGA ATC TTC AAC AAC ATC CAT CAC GAC	977
Thr Thr Ile Asp Arg Asp Tyr Gly Ile Phe Asn Asn Ile His His Asp	
285 290 295	
ATT GGA ACT CAC GTG ATC CAT CAT CTT TTC CCA CAA ATC CCT CAC TAT	1025
Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile Pro His Tyr	
300 305 310	
CAC TTG GTC GAT GCC ACG AGA GCA GCT AAA CAT GTG TTA GGA AGA TAC	1073
His Leu Val Asp Ala Thr Arg Ala Ala Lys His Val Leu Gly Arg Tyr	
315 320 325	
TAC AGA GAG CCG AAG ACG TCA GGA GCA ATA CCG ATT CAC TTG GTG GAG	1121
Tyr Arg Glu Pro Lys Thr Ser Gly Ala Ile Pro Ile His Leu Val Glu	
330 335 340 345	
AGT TTG GTC GCA AGT ATT AAA AAA GAT CAT TAC GTC AGT GAC ACT GGT	1169
Ser Leu Val Ala Ser Ile Lys Lys Asp His Tyr Val Ser Asp Thr Gly	
350 355 360	
GAT ATT GTC TTC TAC GAG ACA GAT CCA GAT CTC TAC GTT TAT GCT TCT	1217
Asp Ile Val Phe Tyr Glu Thr Asp Pro Asp Leu Tyr Val Tyr Ala Ser	
365 370 375	
GAC AAA TCT AAA ATC AAT TAACTTTTCT TCCTAGCTCT ATTAGGAATA	1265
Asp Lys Ser Lys Ile Asn	
380	
AACACTCCTT CTCTTTTACT TATTTGTTTC TGCTTTAAGT TTAAAATGTA CTCGTGAAAC	1325
CTTTTTTTTA TTAATGTATT TACGTTAC	1353

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 383 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Val	Val	Ala	Met	Asp	Gln	Arg	Ser	Asn	Val	Asn	Gly	Asp	Ser	Gly	1	5	10	15
Ala	Arg	Lys	Glu	Glu	Gly	Phe	Asp	Pro	Ser	Ala	Gln	Pro	Pro	Phe	Lys	20	25	30	
Ile	Gly	Asp	Ile	Arg	Ala	Ala	Ile	Pro	Lys	His	Cys	Trp	Val	Lys	Ser	35	40	45	
Pro	Leu	Arg	Ser	Met	Ser	Tyr	Val	Thr	Arg	Asp	Ile	Phe	Ala	Val	Ala	50	55	60	
Ala	Leu	Ala	Met	Ala	Ala	Val	Tyr	Phe	Asp	Ser	Trp	Phe	Leu	Trp	Pro	65	70	75	80
Leu	Tyr	Trp	Val	Ala	Gln	Gly	Thr	Leu	Phe	Trp	Ala	Ile	Phe	Val	Leu	85	90	95	
Gly	His	Asp	Cys	Gly	His	Gly	Ser	Phe	Ser	Asp	Ile	Pro	Leu	Leu	Asn	100	105	110	
Ser	Val	Val	Gly	His	Ile	Leu	His	Ser	Phe	Ile	Leu	Val	Pro	Tyr	His	115	120	125	
Gly	Trp	Arg	Ile	Ser	His	Arg	Thr	His	His	Gln	Asn	His	Gly	His	Val	130	135	140	
Glu	Asn	Asp	Glu	Ser	Trp	Val	Pro	Leu	Pro	Glu	Lys	Leu	Tyr	Lys	Asn	145	150	155	160
Leu	Pro	His	Ser	Thr	Arg	Met	Leu	Arg	Tyr	Thr	Val	Pro	Leu	Pro	Met	165	170	175	
Leu	Ala	Tyr	Pro	Ile	Tyr	Leu	Trp	Tyr	Arg	Ser	Pro	Gly	Lys	Glu	Gly	180	185	190	
Ser	His	Phe	Asn	Pro	Tyr	Ser	Ser	Leu	Phe	Ala	Pro	Ser	Glu	Arg	Lys	195	200	205	
Leu	Ile	Ala	Thr	Ser	Thr	Thr	Cys	Trp	Ser	Ile	Met	Leu	Ala	Thr	Leu	210	215	220	
Val	Tyr	Leu	Ser	Phe	Leu	Val	Asp	Pro	Val	Thr	Val	Leu	Lys	Val	Tyr	225	230	235	240
Gly	Val	Pro	Tyr	Ile	Ile	Phe	Val	Met	Trp	Leu	Asp	Ala	Val	Thr	Tyr	245	250	255	
Leu	His	His	His	Gly	His	Asp	Glu	Lys	Leu	Pro	Trp	Tyr	Arg	Gly	Lys	260	265	270	
Glu	Trp	Ser	Tyr	Leu	Arg	Gly	Gly	Leu	Thr	Thr	Ile	Asp	Arg	Asp	Tyr	275	280	285	

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Gly Ile Phe Asn Asn Ile His His Asp Ile Gly Thr His Val Ile His
 290 295 300
 His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Asp Ala Thr Arg
 305 310 315 320
 Ala Ala Lys His Val Leu Gly Arg Tyr Tyr Arg Glu Pro Lys Thr Ser
 325 330 335
 Gly Ala Ile Pro Ile His Leu Val Glu Ser Leu Val Ala Ser Ile Lys
 340 345 350
 Lys Asp His Tyr Val Ser Asp Thr Gly Asp Ile Val Phe Tyr Glu Thr
 355 360 365
 Asp Pro Asp Leu Tyr Val Tyr Ala Ser Asp Lys Ser Lys Ile Asn
 370 375 380

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCGATGCTG TCGGAATGGA CGATA

25

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTGAGCCA CTATCGACTA CGCGATC

27

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCGATCTCAA GATTACGGAA T

21

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTCCTAATGC AGGAGTCGCA TAAG

24

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGAGTCGCA TAAGGGAG

18

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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GGGAAGTGAA TGGAGAC

17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1645 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 125..1465

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGAAAACACA AGTTTCTCTC ACACACATTA TCTCTTTCTC TATTACCACC ACTCATTTCAT	60
AACAGAAACC CACCAAAAAA TAAAAAGAGA GACTTTTCAC TCTGGGGAGA GAGCTCAAGT	120
TCTA ATG GCG AAC TTG GTC TTA TCA GAA TGT GGT ATA CGA CCT CTC CCC	169
Met Ala Asn Leu Val Leu Ser Glu Cys Gly Ile Arg Pro Leu Pro	
1 5 10 15	
AGA ATC TAC ACA ACA CCC AGA TCC AAT TTC CTC TCC AAC AAC AAC AAA	217
Arg Ile Tyr Thr Thr Pro Arg Ser Asn Phe Leu Ser Asn Asn Asn Lys	
20 25 30	
TTC AGA CCA TCA CTT TCT TCT TCT TCT TAC AAA ACA TCA TCA TCT CCT	265
Phe Arg Pro Ser Leu Ser Ser Ser Ser Tyr Lys Thr Ser Ser Ser Pro	
35 40 45	
CTG TCT TTT GGT CTG AAT TCA CGA GAT GGG TTC ACG AGG AAT TGG GCG	313
Leu Ser Phe Gly Leu Asn Ser Arg Asp Gly Phe Thr Arg Asn Trp Ala	
50 55 60	
TTG AAT GTG AGC ACA CCA TTA ACG ACA CCA ATA TTT GAG GAG TCT CCA	361
Leu Asn Val Ser Thr Pro Leu Thr Thr Pro Ile Phe Glu Glu Ser Pro	
65 70 75	
TTG GAG GAA GAT AAT AAA CAG AGA TTC GAT CCA GGT GCG CCT CCT CCG	409
Leu Glu Glu Asp Asn Lys Gln Arg Phe Asp Pro Gly Ala Pro Pro Pro	
80 85 90 95	
TTC AAT TTA GCT GAT ATT AGA GCA GCT ATA CCT AAG CAT TGT TGG GTT	457
Phe Asn Leu Ala Asp Ile Arg Ala Ala Ile Pro Lys His Cys Trp Val	
100 105 110	
AAG AAT CCA TGG AAG TCT TTG AGT TAT GTC GTC AGA GAC GTC GCT ATC	505
Lys Asn Pro Trp Lys Ser Leu Ser Tyr Val Val Arg Asp Val Ala Ile	
115 120 125	

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GTC TTT GCA TTG GCT GCT GGA GCT GCT TAC CTC AAC AAT TGG ATT GTT	553
Val Phe Ala Leu Ala Ala Gly Ala Ala Tyr Leu Asn Asn Trp Ile Val	
130 135 140	
TGG CCT CTC TAT TGG CTC GCT CAA GGA ACC ATG TTT TGG GCT CTC TTT	601
Trp Pro Leu Tyr Trp Leu Ala Gln Gly Thr Met Phe Trp Ala Leu Phe	
145 150 155	
GTT CTT GGT CAT GAC TGT GGA CAT GGT AGT TTC TCA AAT GAT CCG AAG	649
Val Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asn Asp Pro Lys	
160 165 170 175	
TTG AAC AGT GTG GTC GGT CAT CTT CTT CAT TCC TCA ATT CTG GTC CCA	697
Leu Asn Ser Val Val Gly His Leu Leu His Ser Ser Ile Leu Val Pro	
180 185 190	
TAC CAT GGC TGG AGA ATT AGT CAC AGA ACT CAC CAC CAG AAC CAT GGA	745
Tyr His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly	
195 200 205	
CAT GTT GAG AAT GAC GAA TCT TGG CAT CCT ATG TCT GAG AAA ATC TAC	793
His Val Glu Asn Asp Glu Ser Trp His Pro Met Ser Glu Lys Ile Tyr	
210 215 220	
AAT ACT TTG GAC AAG CCG ACT AGA TTC TTT AGA TTT ACA CTG CCT CTC	841
Asn Thr Leu Asp Lys Pro Thr Arg Phe Phe Arg Phe Thr Leu Pro Leu	
225 230 235	
GTG ATG CTT GCA TAC CCT TTC TAC TTG TGG GCT CGA AGT CCG GGG AAA	889
Val Met Leu Ala Tyr Pro Phe Tyr Leu Trp Ala Arg Ser Pro Gly Lys	
240 245 250 255	
AAG GGT TCT CAT TAC CAT CCA GAC AGT GAC TTG TTC CTC CCT AAA GAG	937
Lys Gly Ser His Tyr His Pro Asp Ser Asp Leu Phe Leu Pro Lys Glu	
260 265 270	
AGA AAG GAT GTC CTC ACT TCT ACT GCT TGT TGG ACT GCA ATG GCT GCT	985
Arg Lys Asp Val Leu Thr Ser Thr Ala Cys Trp Thr Ala Met Ala Ala	
275 280 285	
CTG CTT GTT TGT CTC AAC TTC ACA ATC GGT CCA ATT CAA ATG CTC AAA	1033
Leu Leu Val Cys Leu Asn Phe Thr Ile Gly Pro Ile Gln Met Leu Lys	
290 295 300	
CTT TAT GGA ATT CCT TAC TGG ATA AAT GTA ATG TGG TTG GAC TTT GTG	1081
Leu Tyr Gly Ile Pro Tyr Trp Ile Asn Val Met Trp Leu Asp Phe Val	
305 310 315	
ACT TAC CTG CAT CAC CAT GGT CAT GAA GAT AAG CTT CCT TGG TAC CGT	1129
Thr Tyr Leu His His His Gly His Glu Asp Lys Leu Pro Trp Tyr Arg	
320 325 330 335	
GGC AAG GAG TGG AGT TAC CTG AGA GGA GGA CTT ACA ACA TTG GAT CGT	1177
Gly Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Leu Asp Arg	
340 345 350	

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GAC TAC GGA TTG ATC AAT AAC ATC CAT CAT GAT ATT GGA ACT CAT GTG Asp Tyr Gly Leu Ile Asn Asn Ile His His Asp Ile Gly Thr His Val 355 360 365	1225
ATA CAT CAT CTT TTC CCG CAG ATC CCA CAT TAT CAT CTA GTA GAA GCA Ile His His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Glu Ala 370 375 380	1273
ACA GAA GCA GCT AAA CCA GTA TTA GGG AAG TAT TAC AGG GAG CCT GAT Thr Glu Ala Ala Lys Pro Val Leu Gly Lys Tyr Tyr Arg Glu Pro Asp 385 390 395	1321
AAG TCT GGA CCG TTG CCA TTA CAT TTA CTG GAA ATT CTA GCG AAA AGT Lys Ser Gly Pro Leu Pro Leu His Leu Leu Glu Ile Leu Ala Lys Ser 400 405 410 415	1369
ATA AAA GAA GAT CAT TAC GTG AGC GAC GAA GGA GAA GTT GTA TAC TAT Ile Lys Glu Asp His Tyr Val Ser Asp Glu Gly Glu Val Val Tyr Tyr 420 425 430	1417
AAA GCA GAT CCA AAT CTC TAT GGA GAG GTC AAA GTA AGA GCA GAT TGAATGAAG 1472 Lys Ala Asp Pro Asn Leu Tyr Gly Glu Val Lys Val Arg Ala Asp 435 440 445	
CAGGCTTGAG ATTGAAGTTT TTTCTATTTC AGACCAGCTG ATTTTGTGCT TACTGTATCA	1532
ATTTATTGTG TCACCCACCA GAGAGTTAGT ATCTCTGAAT ACGATCGATC AGATGGAAC	1592
AACAAATTTG TTTGCGATAC TGAAGCTATA TATACCATAA AAAAAAAAAA AAA	1645

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 446 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ala Asn Leu Val Leu Ser Glu Cys Gly Ile Arg Pro Leu Pro Arg 1 5 10 15
Ile Tyr Thr Thr Pro Arg Ser Asn Phe Leu Ser Asn Asn Asn Lys Phe 20 25 30
Arg Pro Ser Leu Ser Ser Ser Ser Tyr Lys Thr Ser Ser Ser Pro Leu 35 40 45
Ser Phe Gly Leu Asn Ser Arg Asp Gly Phe Thr Arg Asn Trp Ala Leu 50 55 60
Asn Val Ser Thr Pro Leu Thr Thr Pro Ile Phe Glu Glu Ser Pro Leu

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65	70	75	80
Glu Glu Asp Asn Lys Gln Arg Phe Asp Pro Gly Ala Pro Pro Pro Phe	85	90	95
Asn Leu Ala Asp Ile Arg Ala Ala Ile Pro Lys His Cys Trp Val Lys	100	105	110
Asn Pro Trp Lys Ser Leu Ser Tyr Val Val Arg Asp Val Ala Ile Val	115	120	125
Phe Ala Leu Ala Ala Gly Ala Ala Tyr Leu Asn Asn Trp Ile Val Trp	130	135	140
Pro Leu Tyr Trp Leu Ala Gln Gly Thr Met Phe Trp Ala Leu Phe Val	145	150	155
Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asn Asp Pro Lys Leu	165	170	175
Asn Ser Val Val Gly His Leu Leu His Ser Ser Ile Leu Val Pro Tyr	180	185	190
His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly His	195	200	205
Val Glu Asn Asp Glu Ser Trp His Pro Met Ser Glu Lys Ile Tyr Asn	210	215	220
Thr Leu Asp Lys Pro Thr Arg Phe Phe Arg Phe Thr Leu Pro Leu Val	225	230	235
Met Leu Ala Tyr Pro Phe Tyr Leu Trp Ala Arg Ser Pro Gly Lys Lys	245	250	255
Gly Ser His Tyr His Pro Asp Ser Asp Leu Phe Leu Pro Lys Glu Arg	260	265	270
Lys Asp Val Leu Thr Ser Thr Ala Cys Trp Thr Ala Met Ala Ala Leu	275	280	285
Leu Val Cys Leu Asn Phe Thr Ile Gly Pro Ile Gln Met Leu Lys Leu	290	295	300
Tyr Gly Ile Pro Tyr Trp Ile Asn Val Met Trp Leu Asp Phe Val Thr	305	310	315
Tyr Leu His His His Gly His Glu Asp Lys Leu Pro Trp Tyr Arg Gly	325	330	335
Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Leu Asp Arg Asp	340	345	350
Tyr Gly Leu Ile Asn Asn Ile His His Asp Ile Gly Thr His Val Ile	355	360	365

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His His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Glu Ala Thr
 370 375 380

Glu Ala Ala Lys Pro Val Leu Gly Lys Tyr Tyr Arg Glu Pro Asp Lys
 385 390 395 400

Ser Gly Pro Leu Pro Leu His Leu Leu Glu Ile Leu Ala Lys Ser Ile
 405 410 415

Lys Glu Asp His Tyr Val Ser Asp Glu Gly Glu Val Val Tyr Tyr Lys
 420 425 430

Ala Asp Pro Asn Leu Tyr Gly Glu Val Lys Val Arg Ala Asp
 435 440 445

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1525 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 61..1368

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGAGAGTGCA AATAGAACGA CAGAGACTTT TTCCTCTTTT CTTCTTGGGA AGAGGCTCCA 60

ATG GCG AGC TCG GTT TTA TCA GAA TGT GGT TTT AGA CCT CTC CCC AGA 108
 Met Ala Ser Ser Val Leu Ser Glu Cys Gly Phe Arg Pro Leu Pro Arg
 1 5 10 15

TTC TAC CCT AAA CAC ACA ACC TCT TTT GCC TCT AAC CCT AAA CCC ACT 156
 Phe Tyr Pro Lys His Thr Thr Ser Phe Ala Ser Asn Pro Lys Pro Thr
 20 25 30

TTC AAA TTC AAT CCA CCA CTT AAA CCT CCT TCT TCT CTT CTC AAT TCC 204
 Phe Lys Phe Asn Pro Pro Leu Lys Pro Pro Ser Ser Leu Leu Asn Ser
 35 40 45

CGA TAT GGA TTC TAC TCT AAA ACC AGG AAC TGG GCA TTG AAT GTG GCA 252
 Arg Tyr Gly Phe Tyr Ser Lys Thr Arg Asn Trp Ala Leu Asn Val Ala
 50 55 60

ACA CCT TTA ACA ACT CTT CAG TCT CCA TCC GAG GAA GAC ACG GAG AGA 300
 Thr Pro Leu Thr Thr Leu Gln Ser Pro Ser Glu Glu Asp Thr Glu Arg
 65 70 75 80

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TTC GAC CCA GGT GCG CCT CCT CCC TTC AAT TTG GCG GAT ATA AGA GCA	348
Phe Asp Pro Gly Ala Pro Pro Pro Phe Asn Leu Ala Asp Ile Arg Ala	
85 90 95	
GCC ATA CCT AAG CAT TGT TGG GTT AAG AAT CCA TGG ATG TCT ATG AGT	396
Ala Ile Pro Lys His Cys Trp Val Lys Asn Pro Trp Met Ser Met Ser	
100 105 110	
TAT GTT GTC AGA GAT GTT GCT ATC GTC TTT GGA TTG GCT GCT GTT GCT	444
Tyr Val Val Arg Asp Val Ala Ile Val Phe Gly Leu Ala Ala Val Ala	
115 120 125	
GCT TAC TTC AAC AAT TGG CTT CTC TGG CCT CTC TAC TGG TTC GCT CAA	492
Ala Tyr Phe Asn Asn Trp Leu Leu Trp Pro Leu Tyr Trp Phe Ala Gln	
130 135 140	
GGA ACC ATG TTC TGG GCT CTC TTT GTC CTT GGC CAT GAC TGC GGA CAT	540
Gly Thr Met Phe Trp Ala Leu Phe Val Leu Gly His Asp Cys Gly His	
145 150 155 160	
GGT AGC TTC TCG AAT GAT CCG AGG CTG AAC AGT GTG GCT GGT CAT CTT	588
Gly Ser Phe Ser Asn Asp Pro Arg Leu Asn Ser Val Ala Gly His Leu	
165 170 175	
CTT CAT TCC TCA ATT CTG GTC CCT TAC CAT GGC TGG AGG ATT AGC CAC	636
Leu His Ser Ser Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His	
180 185 190	
AGA ACT CAC CAC CAG AAC CAT GGT CAT GTC GAG AAT GAC GAA TCA TGG	684
Arg Thr His His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp	
195 200 205	
CAT CCT TTG CCT GAA AGC ATC TAC AAG AAT TTG GAA AAG ACG ACT CAA	732
His Pro Leu Pro Glu Ser Ile Tyr Lys Asn Leu Glu Lys Thr Thr Gln	
210 215 220	
ATG TTT AGG TTT ACA CTG CCT TTT CCA ATG CTC GCA TAC CCT TTC TAC	780
Met Phe Arg Phe Thr Leu Pro Phe Pro Met Leu Ala Tyr Pro Phe Tyr	
225 230 235 240	
TTG TGG AAC AGA AGT CCA GGG AAA CAA GGT TCT CAT TAT CAT CCG GAC	828
Leu Trp Asn Arg Ser Pro Gly Lys Gln Gly Ser His Tyr His Pro Asp	
245 250 255	
AGT GAC TTG TTT CTT CCA AAA GAG AAG AAA GAT GTT CTG ACA TCA ACT	876
Ser Asp Leu Phe Leu Pro Lys Glu Lys Lys Asp Val Leu Thr Ser Thr	
260 265 270	
GCC TGT TGG ACT GCA ATG GCT GCT TTG CTT GTT TGT CTC AAC TTT GTC	924
Ala Cys Trp Thr Ala Met Ala Ala Leu Leu Val Cys Leu Asn Phe Val	
275 280 285	
ATG GGT CCA ATC CAG ATG CTC AAA CTA TAT GGC ATC CCT TAT TGG ATA	972
Met Gly Pro Ile Gln Met Leu Lys Leu Tyr Gly Ile Pro Tyr Trp Ile	
290 295 300	

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TTT GTA ATG TGG TTG GAC TTC GTC ACT TAC TTG CAC CAC CAT GGA CAT Phe Val Met Trp Leu Asp Phe Val Thr Tyr Leu His His His Gly His 305 310 315 320	1020
GAA GAC AAG CTC CCT TGG TAT CGT GGA AAG GAA TGG AGT TAC CTG AGA Glu Asp Lys Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg 325 330 335	1068
GGA GGG CTC ACA ACA TTA GAT CGT GAC TAC GGA TGG ATC AAT AAC ATC Gly Gly Leu Thr Thr Leu Asp Arg Asp Tyr Gly Trp Ile Asn Asn Ile 340 345 350	1116
CAC CAC GAT ATT GGA ACT CAT GTG ATA CAT CAT CTT TTC CCG CAG ATC His His Asp Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile 355 360 365	1164
CCA CAT TAT CAT CTA GTA GAA GCA ACA GAA GCA GCT AAA CCA GTA CTA Pro His Tyr His Leu Val Glu Ala Thr Glu Ala Ala Lys Pro Val Leu 370 375 380	1212
GGA AAG TAC TAC AGA GAA CCG AAA AAC TCT GGA CCT CTG CCA CTT CAC Gly Lys Tyr Tyr Arg Glu Pro Lys Asn Ser Gly Pro Leu Pro Leu His 385 390 395 400	1260
TTA CTG GGA AGC CTC ATA AAG AGT ATG AAA CAA GAC CAT TTC GTA AGC Leu Leu Gly Ser Leu Ile Lys Ser Met Lys Gln Asp His Phe Val Ser 405 410 415	1308
GAT ACA GGA GAT GTC GTG TAC TAT GAG GCA GAT CCA AAA CTC AAT GGA Asp Thr Gly Asp Val Val Tyr Tyr Glu Ala Asp Pro Lys Leu Asn Gly 420 425 430	1356
CAA AGA ACA TGAGGACATA CTGCAGTGAA CCAGGCAGAC AAGTTACATA Gln Arg Thr 435	1405
AATTCATCTT GGCCCATTC A TTATGTTCTT TTTGTTTGG TGTAAGCCT TTTCCGAGATT	1465
AAAAAAGCAT TAATTTGTAG AAACCTGTGG TAAACTCTC GATCAAATGA AATAAGATAT	1525

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 435 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met	Ala	Ser	Ser	Val	Leu	Ser	Glu	Cys	Gly	Phe	Arg	Pro	Leu	Pro	Arg
1					5				10					15	

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Phe Tyr Pro Lys His Thr Thr Ser Phe Ala Ser Asn Pro Lys Pro Thr
 20 25 30
 Phe Lys Phe Asn Pro Pro Leu Lys Pro Pro Ser Ser Leu Leu Asn Ser
 35 40 45
 Arg Tyr Gly Phe Tyr Ser Lys Thr Arg Asn Trp Ala Leu Asn Val Ala
 50 55 60
 Thr Pro Leu Thr Thr Leu Gln Ser Pro Ser Glu Glu Asp Thr Glu Arg
 65 70 75 80
 Phe Asp Pro Gly Ala Pro Pro Pro Phe Asn Leu Ala Asp Ile Arg Ala
 85 90 95
 Ala Ile Pro Lys His Cys Trp Val Lys Asn Pro Trp Met Ser Met Ser
 100 105 110
 Tyr Val Val Arg Asp Val Ala Ile Val Phe Gly Leu Ala Ala Val Ala
 115 120 125
 Ala Tyr Phe Asn Asn Trp Leu Leu Trp Pro Leu Tyr Trp Phe Ala Gln
 130 135 140
 Gly Thr Met Phe Trp Ala Leu Phe Val Leu Gly His Asp Cys Gly His
 145 150 155 160
 Gly Ser Phe Ser Asn Asp Pro Arg Leu Asn Ser Val Ala Gly His Leu
 165 170 175
 Leu His Ser Ser Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His
 180 185 190
 Arg Thr His His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp
 195 200 205
 His Pro Leu Pro Glu Ser Ile Tyr Lys Asn Leu Glu Lys Thr Thr Gln
 210 215 220
 Met Phe Arg Phe Thr Leu Pro Phe Pro Met Leu Ala Tyr Pro Phe Tyr
 225 230 235 240
 Leu Trp Asn Arg Ser Pro Gly Lys Gln Gly Ser His Tyr His Pro Asp
 245 250 255
 Ser Asp Leu Phe Leu Pro Lys Glu Lys Lys Asp Val Leu Thr Ser Thr
 260 265 270
 Ala Cys Trp Thr Ala Met Ala Ala Leu Leu Val Cys Leu Asn Phe Val
 275 280 285
 Met Gly Pro Ile Gln Met Leu Lys Leu Tyr Gly Ile Pro Tyr Trp Ile
 290 295 300
 Phe Val Met Trp Leu Asp Phe Val Thr Tyr Leu His His His Gly His
 305 310 315 320

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Glu	Asp	Lys	Leu	Pro	Trp	Tyr	Arg	Gly	Lys	Glu	Trp	Ser	Tyr	Leu	Arg
325								330						335	
Gly	Gly	Leu	Thr	Thr	Leu	Asp	Arg	Asp	Tyr	Gly	Trp	Ile	Asn	Asn	Ile
340							345				350				
His	His	Asp	Ile	Gly	Thr	His	Val	Ile	His	His	Leu	Phe	Pro	Gln	Ile
355							360			365					
Pro	His	Tyr	His	Leu	Val	Glu	Ala	Thr	Glu	Ala	Ala	Lys	Pro	Val	Leu
370				375				380							
Gly	Lys	Tyr	Tyr	Arg	Glu	Pro	Lys	Asn	Ser	Gly	Pro	Leu	Pro	Leu	His
385				390				395				400			
Leu	Leu	Gly	Ser	Leu	Ile	Lys	Ser	Met	Lys	Gln	Asp	His	Phe	Val	Ser
405					410					415					
Asp	Thr	Gly	Asp	Val	Val	Tyr	Tyr	Glu	Ala	Asp	Pro	Lys	Leu	Asn	Gly
420							425				430				
Gln	Arg	Thr													
435															

(2) INFORMATION FOR SEQ ID NO:13:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAYATHMGNG CNGCNATHCC

20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCNATHCCNA ARCA YTG

17

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(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AARCAITGYT GGGTNA

17

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGGTYYTNT GGCCNYTNTA YTG

24

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGGTYYTNT GGCCN

15

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGCCNYTNT AYTGG

15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGGGTNGCNC ARGGNAC

17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTYGTNYTNG GNCAYGA

17

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTNYTNGGNC AYGAYTG

17

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(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGNCAYGAYT GYGGNCA

17

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGYGGNCAYG GNWSNTT

17

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCNTAYCAYG GNTGG

15

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAYGGNTGGM GNATHWSNCA

20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TGGMGNATHT CNCAYMGNAC NCAYCA

26

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGGMGNATHA GYCAYMGNAC NCAYCA

26

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TGGMGNATHW SNCAY

15

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(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CAYMGNACNC AYCA

15

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GARAAYGAYG ARWSNTGG

18

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAYGARWSNT GGGTNCC

17

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

NGTNACNGCR TCNARCCA

18

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

RTGRTGNARR TANGT

15

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ARNCCNCCNC KNARRTARCT CCA

23

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ARNCCNCCNC KNARRTANGA CCA

23

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(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

RTCNCCKRTCD ATNGTNGTNA

20

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

RTARTCNCKR TCDATNGT

18

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

RTGNGTNCCD ATRTCRTG

18

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

NARRTGRTGD ATNACRTG

18

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

DATYTGNGGR AANARRTGRT G

21

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGDATYTGNG GRAANARRTG

20

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

RTARTGNGGD ATYTGNGGRA ANA

23

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(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Asp Ile Arg Ala Ala Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Ala Ile Pro Lys His Cys
1 5

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Lys His Cys Trp Val Lys
1 5

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Trp Phe Leu Trp Pro Leu Tyr Trp
1 5

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Trp Phe Leu Trp Pro
1 5

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Trp Pro Leu Tyr Trp
1 5

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Trp Val Ala Gln Gly Thr
1 5

(2) INFORMATION FOR SEQ ID NO:50:

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- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Trp Val Ala Gln Gly Thr
1 5

(2) INFORMATION FOR SEQ ID NO:51:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Val Leu Gly His Asp Cys
1 5

(2) INFORMATION FOR SEQ ID NO:52:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:53:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Cys Gly His Gly Ser Phe
1 5

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Pro Tyr His Gly Trp
1 5

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

His Gly Trp Arg Ile Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Trp Arg Ile Ser His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Trp Arg Ile Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Glu Asn Asp Glu Ser Trp
1 5

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Asp Glu Ser Trp Val Pro
1 5

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Trp Leu Asp Ala Val Thr
1 5

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Thr Tyr Leu His His
1 5

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Trp Ser Tyr Leu Arg Gly Gly Leu
1 5

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Leu Thr Thr Ile Asp Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Thr Ile Asp Arg Asp Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

His Asp Ile Gly Thr His
1 5

(2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

His Val Ile His His Leu
1 5

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

His His Leu Phe Pro Gln Ile
1 5

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

His Leu Phe Pro Gln Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Leu Phe Pro Gln Ile Pro His Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 1670 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(1x) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 46..1302

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CAA	ACT	TCT	TCT	CGG	GGG	TCG	CTT	TCT	TCT	GC	ATT	TTT	CT	GCT	TCC	54
Met Ala Ser																
1																
AGA	ATT	GCT	GAT	TCT	CTC	TTC	GCC	TTC	ACG	GGC	CCA	CAG	CAA	TGT	CTT	102
Arg	Ile	Ala	Asp	Ser	Leu	Phe	Ala	Phe	Thr	Gly	Pro	Gln	Gln	Cys	Leu	
5						10				15						
CCT	AGG	GTT	CCT	AAG	CTT	GCT	GCT	TCT	TCT	GCT	CGT	GTT	TCT	CCT	GGT	150
Pro	Arg	Val	Pro	Lys	Leu	Ala	Ala	Ser	Ser	Ala	Arg	Val	Ser	Pro	Gly	
20				25						30				35		
GTA	TAT	GCT	GTG	AAG	CCG	ATT	GAT	CTT	CTG	TTA	AAA	GGA	CGA	ACT	CAT	198
Val	Tyr	Ala	Val	Lys	Pro	Ile	Asp	Leu	Leu	Leu	Lys	Gly	Arg	Thr	His	
				40				45						50		
CGA	AGT	AGA	AGA	TGT	GTA	GCT	CCT	GTG	AAA	AGG	AGA	ATT	GGA	TGT	ATC	246
Arg	Ser	Arg	Arg	Cys	Val	Ala	Pro	Val	Lys	Arg	Arg	Ile	Gly	Cys	Ile	
		55						60				65				
AAA	GCG	GTG	GCT	GCT	CCA	GTT	GCA	CCG	CCT	TCA	GCT	GAC	AGT	GCA	GAA	294
Lys	Ala	Val	Ala	Ala	Pro	Val	Ala	Pro	Pro	Ser	Ala	Asp	Ser	Ala	Glu	
70						75						80				
GAC	AGG	GAA	CAG	TTA	GCA	GAA	AGC	TAT	GGA	TTC	AGA	CAA	ATT	GGA	GAA	342
Asp	Arg	Glu	Gln	Leu	Ala	Glu	Ser	Tyr	Gly	Phe	Arg	Gln	Ile	Gly	Glu	
85						90				95						
GAT	CTT	CCT	GAG	AAT	GTC	ACC	TTA	AAA	GAT	ATC	ATG	GAT	ACA	CTT	CCC	390
Asp	Leu	Pro	Glu	Asn	Val	Thr	Leu	Lys	Asp	Ile	Met	Asp	Thr	Leu	Pro	
100				105						110				115		
AAA	GAG	GTG	TTT	GAG	ATT	GAT	GAT	CTG	AAA	GCT	TTG	AAG	TCT	GTG	TTG	438
Lys	Glu	Val	Phe	Glu	Ile	Asp	Asp	Leu	Lys	Ala	Leu	Lys	Ser	Val	Leu	
				120				125						130		
ATA	TCT	GTG	ACT	TCA	TAC	ACT	TTG	GGG	CTC	TTC	ATG	ATT	GCA	AAA	TCG	486
Ile	Ser	Val	Thr	Ser	Tyr	Thr	Leu	Gly	Leu	Phe	Met	Ile	Ala	Lys	Ser	
		135						140				145				
CCG	TGG	TAT	CTG	CTA	CCG	TTG	GCT	TGG	GCA	TGG	ACA	GGA	ACT	GCA	ATT	534

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Pro Trp Tyr Leu Leu Pro Leu Ala Trp Ala Trp Thr Gly Thr Ala Ile	
150 155 160	
ACC GGG TTC TTT GTG ATA GGT CAT GAT TGT GCA CAT AAG TCA TTT TCA	582
Thr Gly Phe Phe Val Ile Gly His Asp Cys Ala His Lys Ser Phe Ser	
165 170 175	
AAG AAC AAA TTG GTG GAA GAC ATT GTG GGT ACT CTC GCC TTC CTA CCA	630
Lys Asn Lys Leu Val Glu Asp Ile Val Gly Thr Leu Ala Phe Leu Pro	
180 185 190 195	
CTT GTC TAC CCA TAT GAG CCA TGG CGG TTT AAG CAC GAC CGC CAT CAC	678
Leu Val Tyr Pro Tyr Glu Pro Trp Arg Phe Lys His Asp Arg His His	
200 205 210	
GCC AAA ACC AAC ATG TTA CTT CAT GAC ACA GCT TGG CAG CCA GTT CCG	726
Ala Lys Thr Asn Met Leu Leu His Asp Thr Ala Trp Gln Pro Val Pro	
215 220 225	
CCA GAG GAG TTT GAG TCA TCA CCC GTG ATG AGA AAG GCA ATC ATT TTT	774
Pro Glu Glu Phe Glu Ser Ser Pro Val Met Arg Lys Ala Ile Ile Phe	
230 235 240	
GGA TAT GGC CCA ATT AGA CCT TGG TTG TCC ATA GCT CAC TGG GTG AAC	822
Gly Tyr Gly Pro Ile Arg Pro Trp Leu Ser Ile Ala His Trp Val Asn	
245 250 255	
TGG CAC TTC AAT CTG AAA AAG TTC AGA GCG AGC GAG GTG AAT AGG GTG	870
Trp His Phe Asn Leu Lys Lys Phe Arg Ala Ser Glu Val Asn Arg Val	
260 265 270 275	
AAG ATA AGT TTG GCT TGT GTT TTC GCC TTC ATG GCC GTT GGG TGG CCA	918
Lys Ile Ser Leu Ala Cys Val Phe Ala Phe Met Ala Val Gly Trp Pro	
280 285 290	
CTG ATC GTA TAC AAA GTT GGT ATA TTG GGA TGG GTA AAA TTC TGG TTA	966
Leu Ile Val Tyr Lys Val Gly Ile Leu Gly Trp Val Lys Phe Trp Leu	
295 300 305	
ATG CCA TGG TTG GGC TAT CAC TTC TGG ATG AGC ACA TTC ACA ATG GTT	1014
Met Pro Trp Leu Gly Tyr His Phe Trp Met Ser Thr Phe Thr Met Val	
310 315 320	
CAT CAT ACG GCT CCG CAT ATA CCT TTC AAG CCT GCG GAT GAG TGG AAC	1062
His His Thr Ala Pro His Ile Pro Phe Lys Pro Ala Asp Glu Trp Asn	
325 330 335	
GCG GCT CAG GCC CAG CTG AAT GGA ACT GTT CAT TGT GAC TAC CCT AGT	1110
Ala Ala Gln Ala Gln Leu Asn Gly Thr Val His Cys Asp Tyr Pro Ser	
340 345 350 355	
TGG ATT GAA ATT CTC TGC CAT GAT ATC AAC GTT CAC ATC CCG CAT CAT	1158
Trp Ile Glu Ile Leu Cys His Asp Ile Asn Val His Ile Pro His His	
360 365 370	
ATT AGC CCA AGA ATA CCG AGC TAC AAT CTC CGT GCA GCT CAT GAG TCT	1206

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Ile Ser Pro Arg Ile Pro Ser Tyr Asn Leu Arg Ala Ala His Glu Ser
 375 380 385

ATA CAA GAG AAC TGG GGA AAG TAT ACA AAC TTG GCT ACA TGG AAC TGG 1254
 Ile Gln Glu Asn Trp Gly Lys Tyr Thr Asn Leu Ala Thr Trp Asn Trp
 390 395 400

CGA TTG ATG AAG ACG ATA ATG ACT GTG TGT CAT GTC TAT GAC AAA TAGGAGAACT
 1309

Arg Leu Met Lys Thr Ile Met Thr Val Cys His Val Tyr Asp Lys
 405 410 415

ACATTCCTTT TGACCGGTTA GCCCCTGAAG AATCTCAGCC AATAACCTTC CTCAGAAAT 1369

CAATGCCTAA CTACACAGCC TGATTCGCCA TGGTCTCAAA CTAGTCTTTT GAAATCTCAA 1429

TATCTTTTTG CAGTCGCCGA TGTTATATGT AAGCTTTCCA AGCGATGAGC TTCTCTAACA 1489

CTTCACCAAC GCTTTATACT GTTATCTTCT TTCCAATCTT ATCAGAAGAG AGAACTGGT 1549

CAAATTATCT GAGCGATTGC AATTCTTTTA TCAGTTTCTT AGCTATAAGA AGATTGAACA 1609

GTCTATATAG TTTGCAATGT ACTGTAATGT GATGAAAATT TAGTTGATGA GAAAAAAAAA 1669

A 1670

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 418 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Met Ala Ser Arg Ile Ala Asp Ser Leu Phe Ala Phe Thr Gly Pro Gln
 1 5 10 15

Gln Cys Leu Pro Arg Val Pro Lys Leu Ala Ala Ser Ser Ala Arg Val
 20 25 30

Ser Pro Gly Val Tyr Ala Val Lys Pro Ile Asp Leu Leu Leu Lys Gly
 35 40 45

Arg Thr His Arg Ser Arg Arg Cys Val Ala Pro Val Lys Arg Arg Ile
 50 55 60

Gly Cys Ile Lys Ala Val Ala Ala Pro Val Ala Pro Pro Ser Ala Asp
 65 70 75 80

Ser Ala Glu Asp Arg Glu Gln Leu Ala Glu Ser Tyr Gly Phe Arg Gln
 85 90 95

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Ile Gly Glu Asp Leu Pro Glu Asn Val Thr Leu Lys Asp Ile Met Asp
 100 105 110
 Thr Leu Pro Lys Glu Val Phe Glu Ile Asp Asp Leu Lys Ala Leu Lys
 115 120 125
 Ser Val Leu Ile Ser Val Thr Ser Tyr Thr Leu Gly Leu Phe Met Ile
 130 135 140
 Ala Lys Ser Pro Trp Tyr Leu Leu Pro Leu Ala Trp Ala Trp Thr Gly
 145 150 155 160
 Thr Ala Ile Thr Gly Phe Phe Val Ile Gly His Asp Cys Ala His Lys
 165 170 175
 Ser Phe Ser Lys Asn Lys Leu Val Glu Asp Ile Val Gly Thr Leu Ala
 180 185 190
 Phe Leu Pro Leu Val Tyr Pro Tyr Glu Pro Trp Arg Phe Lys His Asp
 195 200 205
 Arg His His Ala Lys Thr Asn Met Leu Leu His Asp Thr Ala Trp Gln
 210 215 220
 Pro Val Pro Pro Glu Glu Phe Glu Ser Ser Pro Val Met Arg Lys Ala
 225 230 235 240
 Ile Ile Phe Gly Tyr Gly Pro Ile Arg Pro Trp Leu Ser Ile Ala His
 245 250 255
 Trp Val Asn Trp His Phe Asn Leu Lys Lys Phe Arg Ala Ser Glu Val
 260 265 270
 Asn Arg Val Lys Ile Ser Leu Ala Cys Val Phe Ala Phe Met Ala Val
 275 280 285
 Gly Trp Pro Leu Ile Val Tyr Lys Val Gly Ile Leu Gly Trp Val Lys
 290 295 300
 Phe Trp Leu Met Pro Trp Leu Gly Tyr His Phe Trp Met Ser Thr Phe
 305 310 315 320
 Thr Met Val His His Thr Ala Pro His Ile Pro Phe Lys Pro Ala Asp
 325 330 335
 Glu Trp Asn Ala Ala Gln Ala Gln Leu Asn Gly Thr Val His Cys Asp
 340 345 350
 Tyr Pro Ser Trp Ile Glu Ile Leu Cys His Asp Ile Asn Val His Ile
 355 360 365
 Pro His His Ile Ser Pro Arg Ile Pro Ser Tyr Asn Leu Arg Ala Ala
 370 375 380
 His Glu Ser Ile Gln Glu Asn Trp Gly Lys Tyr Thr Asn Leu Ala Thr
 385 390 395 400

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Trp Asn Trp Arg Leu Met Lys Thr Ile Met Thr Val Cys His Val Tyr
405 410 415

Asp Lys

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Claims:

1. A genetically transformed plant which has an elevated
linolenic acid content comprising a recombinant, double-stranded DNA
5 molecule comprising
- (i) a promoter that functions in plant cells to cause
the production of an RNA sequence, said promoter
operably linked to;
 - 10 (ii) a structural coding sequence that causes the
production of an RNA sequence that encodes a linoleic
acid desaturase activity; and
 - (iii) a 3' non-translated region that functions in plant
cells to promote polyadenylation to the 3' end of said RNA
sequence.
- 15 2. The plant of claim 1 in which the linoleic acid desaturase
activity is from plants.
3. The plant of claim 1 in which the linoleic acid desaturase
activity is from fungi, algae or bacteria.
4. The plant of claim 1 in which the structural coding
20 sequence of (ii) is taken from SEQ. ID NO:1.
5. The plant of claim 1 in which the structural coding
sequence of (ii) is taken from SEQ. ID NO:9.
6. The plant of claim 1 in which the structural coding
sequence of (ii) is taken from SEQ. ID NO:11.
- 25 7. The plant of claim 1 in which the promoter of (i) is an
endogenous plant linoleic acid desaturase promoter.
8. A genetically transformed plant which has a reduced
linolenic acid content, comprising a recombinant, double-stranded DNA
molecule comprising

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(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

5 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

10 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

9. The plant of claim 8 in which the linoleic acid desaturase enzyme is from plants.

10. The plant of claim 8 in which the linoleic acid desaturase enzyme is from fungi, algae or bacteria.

15 11. The plant of claim 8 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:1.

12. The plant of claim 8 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:9.

20 13. The plant of claim 8 in which the structural coding sequence of (ii) is taken from SEQ. 8 ID NO:11.

14. The plant of claim 8 in which the promoter of (i) is an endogenous plant linoleic acid desaturase promoter.

25 15. A genetically transformed plant which has an improved resistance to low temperatures comprising a recombinant, double-stranded DNA molecule comprising

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

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- (ii) a structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity; and
- (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.
- 5
16. A genetically transformed plant which has an elevated ability to respond to pathogens, comprising a recombinant, double-stranded DNA molecule comprising
- 10 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;
- (ii) a structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity; and
- 15 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.
17. A seed produced from genetically transformed plant where
- 20 said seed has an linolenic acid content suitable for use as a source of linolenic acid, said plant comprising a recombinant, double-stranded DNA molecule comprising
- (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;
- 25 (ii) a structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity; and

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(iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

18. The seed of claim 17 where said plant is selected from the group consisting of soybean and rapeseed.

19. A genetically transformed plant which has a linolenic acid content of less than about 3%, said plant comprising a recombinant, double-stranded DNA molecule comprising

10 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

(ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

15 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

20. A genetically transformed plant which has an increased oleic acid content, comprising a recombinant, double-stranded DNA molecule comprising

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

25 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a oleic acid desaturase activity in said plant; and

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(iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

21. A genetically transformed plant which has an increased
5 oleic acid content, comprising a recombinant, double-stranded DNA molecule comprising

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

10 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

15 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

22. A method of producing a genetically transformed plant which has an elevated linolenic acid content, comprising

20 (a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising:

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

25 (ii) a structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity; and

(iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence;

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- (b) obtaining transformed plant cells; and
- (c) regenerating from the transformed plant cells genetically transformed plants which have an elevated linolenic acid content.

5 23. The method of claim 22 in which the linoleic acid desaturase enzyme is from plants.

 24. The method of claim 22 in which the linoleic acid desaturase enzyme is from fungi, algae or bacteria.

 25. The method of claim 22 in which the structural coding
10 sequence of (ii) is taken from SEQ. ID NO:1.

 26. The method of claim 22 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:9.

 27. The method of claim 22 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:11.

15 28. The plant of claim 22 in which the promoter of (i) is an endogenous plant linoleic acid desaturase promoter.

 29. A method of producing a genetically transformed plant which has a reduced linolenic acid content, comprising

20 (a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising:

 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

25 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

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- (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence
- (b) obtaining transformed plant cells; and
- 5 (c) regenerating from the transformed plant cells genetically transformed plants which have a reduced linolenic acid content.
30. The method of claim 29 in which the linoleic acid desaturase enzyme is from plants.
- 10 31. The method of claim 29 in which the linoleic acid desaturase enzyme is from fungi, algae or bacteria.
32. The method of claim 29 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:1.
33. The method of claim 29 in which the structural coding
- 15 sequence of (ii) is taken from SEQ. ID NO:9.
34. The method of claim 29 in which the structural coding sequence of (ii) is taken from SEQ. ID NO:11.
35. The plant of claim 29 in which the promoter of (i) is an endogenous plant linoleic acid desaturase promoter.
- 20 36. A method of producing a genetically transformed plant which has an increased oleic acid content, comprising
- (a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising:
- 25 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;
- (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a

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gene that encodes a linoleic acid desaturase activity in said plant; and

(iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence

(b) obtaining transformed plant cells; and

(c) regenerating from the transformed plant cells genetically transformed plants which have an increased oleic acid content.

10 37. A recombinant, double-stranded DNA molecule comprising in sequence:

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

15 (ii) a structural coding sequence that causes the production of an RNA sequence that encodes a linoleic acid desaturase activity; and

20 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

38. A recombinant, double-stranded DNA molecule comprising in sequence:

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

25 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

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(iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

5 39. A plant cell comprising a recombinant, double-stranded DNA molecule comprising in sequence:

(i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

10 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a linoleic acid desaturase activity in said plant; and

15 (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence.

40. A method of producing a genetically transformed plant which has an increased oleic acid content, comprising

(a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising:

20 (i) a promoter that functions in plant cells to cause the production of an RNA sequence, said promoter operably linked to;

25 (ii) a DNA sequence that causes the production of an RNA sequence that is in antisense orientation to at least a portion of a gene that encodes a oleic acid desaturase activity in said plant; and

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- 5
- (iii) a 3' non-translated region that functions in plant cells to promote polyadenylation to the 3' end of said RNA sequence
 - (b) obtaining transformed plant cells; and
 - (c) regenerating from the transformed plant cells genetically transformed plants which have an increased oleic acid content.

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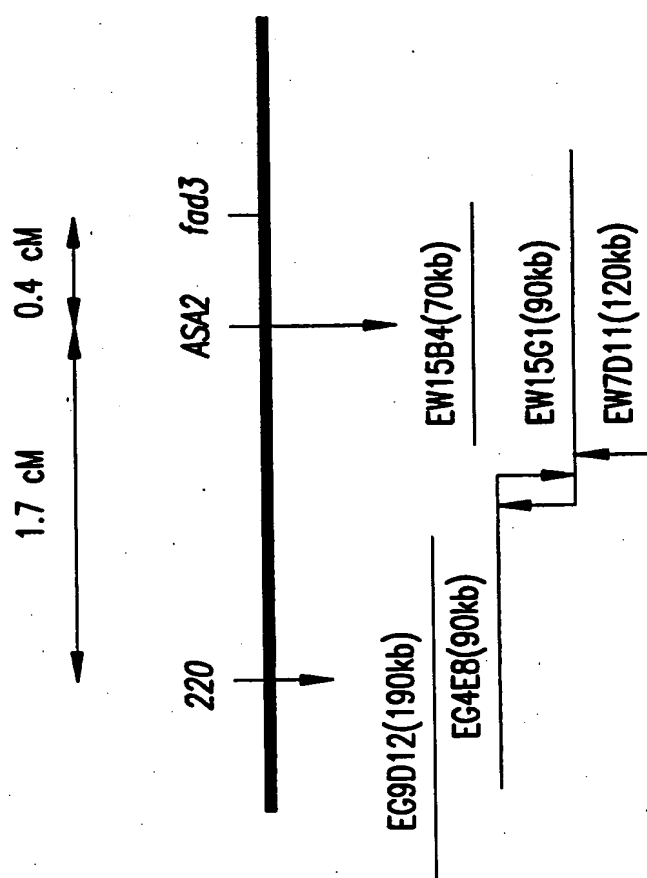


FIG.1

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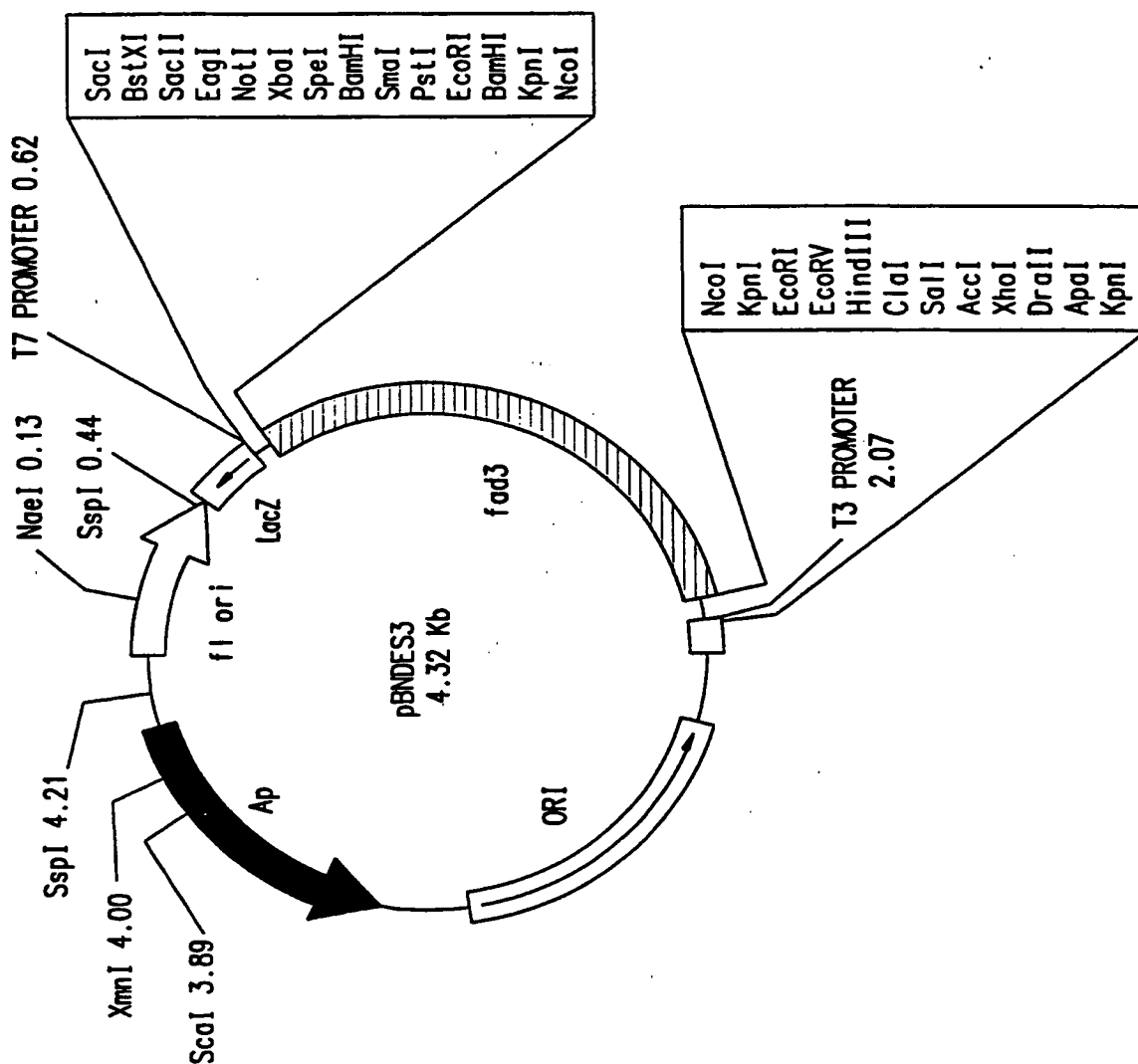


FIG.2

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AATCCATCAA ACCTTTATTC ACCACATTTT ACTGAAAGGC CACACATCTA GAGAGAGAAA	60
CTTCGTCCAA ATCTCTCTCT CCAGCG ATG GTT GTT GCT ATG GAC CAG CGC AGC	113
Met Val Val Ala Met Asp Gln Arg Ser	
1 5	
AAT GTT AAC GGA GAT TCC GGT GCC CGG AAG GAA GAA GGG TTT GAT CCA	161
Asn Val Asn Gly Asp Ser Gly Ala Arg Lys Glu Glu Gly Phe Asp Pro	
10 15 20 25	
AGC GCA CAA CCA CCG TTT AAG ATC GGA GAT ATA AGG GCG GCG ATT CCT	209
Ser Ala Gln Pro Pro Phe Lys Ile Gly Asp Ile Arg Ala Ala Ile Pro	
30 35 40	
AAG CAT TGC TGG GTG AAG AGT CCT TTG AGA TCT ATG AGC TAC GTC ACC	257
Lys His Cys Trp Val Lys Ser Pro Leu Arg Ser Met Ser Tyr Val Thr	
45 50 55	
AGA GAC ATT TTC GCC GTC CGG GCT CTG GCC ATG GCC GCC GTG TAT TTT	305
Arg Asp Ile Phe Ala Val Ala Ala Leu Ala Met Ala Ala Val Tyr Phe	
60 65 70	
GAT AGC TGG TTC CTC TGG CCA CTC TAC TGG GTT GCC CAA GGA ACC CTT	353
Asp Ser Trp Phe Leu Trp Pro Leu Tyr Trp Val Ala Gln Gly Thr Leu	
75 80 85	
TTC TGG GCC ATC TTC GTT CTT GGC CAC GAC TGT GGA CAT GGG AGT TTC	401
Phe Trp Ala Ile Phe Val Leu Gly His Asp Cys Gly His Gly Ser Phe	
90 95 100 105	
TCA GAC ATT CCT CTG CTG AAC AGT GTG GTT GGT CAC ATT CTT CAT TCA	449
Ser Asp Ile Pro Leu Leu Asn Ser Val Val Gly His Ile Leu His Ser	
110 115 120	
TTC ATC CTC GTT CCT TAC CAT GGT TGG AGA ATA AGC CAT CGG ACA CAC	497
Phe Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His Arg Thr His	
125 130 135	
CAC CAG AAC CAT GGC CAT GTT GAA AAC GAC GAG TCT TGG GTT CCG TTG	545
His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp Val Pro Leu	
140 145 150	

FIG.3a

 RECTIFIED SHEET (RULE 91)
 ISA/EP

CCA GAA AAG TTG TAC AAG AAC TTG CCC CAT AGT ACT CGG ATG CTC AGA Pro Glu Lys Leu Tyr Lys Asn Leu Pro His Ser Thr Arg Met Leu Arg 155 160 165	593
TAC ACT GTC CCT CTG CCC ATG CTC GCT TAC CCG ATC TAT CTG TGG TAC Tyr Thr Val Pro Leu Pro Met Leu Ala Tyr Pro Ile Tyr Leu Trp Tyr 170 175 180 185	641
AGA AGT CCT GGA AAA GAA GGG TCA CAT TTT AAC CCA TAC AGT AGT TTA Arg Ser Pro Gly Lys Glu Gly Ser His Phe Asn Pro Tyr Ser Ser Leu 190 195 200	689
TTT GCT CCA AGC GAG AGG AAG CTT ATT GCA ACT TCA ACT ACT TGC TGG Phe Ala Pro Ser Glu Arg Lys Leu Ile Ala Thr Ser Thr Thr Cys Trp 205 210 215	737
TCC ATA ATG TTG GCC ACT CTT GTT TAT CTA TCG TTC CTC GTT GAT CCA Ser Ile Met Leu Ala Thr Leu Val Tyr Leu Ser Phe Leu Val Asp Pro 220 225 230	785
GTC ACA GTT CTC AAA GTC TAT GGC GTT CCT TAC ATT ATC TTT GTG ATG Val Thr Val Leu Lys Val Tyr Gly Val Pro Tyr Ile Ile Phe Val Met 235 240 245	833
TGG TTG GAC GCT GTC ACG TAC TTG CAT CAT CAT GGT CAC GAT GAG AAG Trp Leu Asp Ala Val Thr Tyr Leu His His His Gly His Asp Glu Lys 250 255 260 265	881
TTG CCT TGG TAC AGA GGC AAG GAA TGG AGT TAT TTA CGT GGA GGA TTA Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu 270 275 280	929
ACA ACT ATT GAT AGA GAT TAC GGA ATC TTC AAC AAC ATC CAT CAC GAC Thr Thr Ile Asp Arg Asp Tyr Gly Ile Phe Asn Asn Ile His His Asp 285 290 295	977
ATT GGA ACT CAC GTG ATC CAT CAT CTT TTC CCA CAA ATC CCT CAC TAT Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile Pro His Tyr 300 305 310	1025

FIG.3b

RECTIFIED SHEET (RULE 91)
ISAVEP

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CAC TTG GTC GAT GCC ACG AGA GCA GCT AAA CAT GTG TTA GGA AGA TAC His Leu Val Asp Ala Thr Arg Ala Ala Lys His Val Leu Gly Arg Tyr 315 320 325	1073
TAC AGA GAG CCG AAG ACG TCA GGA GCA ATA CCG ATT CAC TTG GTG GAG Tyr Arg Glu Pro Lys Thr Ser Gly Ala Ile Pro Ile His Leu Val Glu 330 335 340 345	1121
AGT TTG GTC GCA AGT ATT AAA AAA GAT CAT TAC GTC AGT GAC ACT GGT Ser Leu Val Ala Ser Ile Lys Lys Asp His Tyr Val Ser Asp Thr Gly 350 355 360	1169
GAT ATT GTC TTC TAC GAG ACA GAT CCA GAT CTC TAC GTT TAT GCT TCT Asp Ile Val Phe Tyr Glu Thr Asp Pro Asp Leu Tyr Val Tyr Ala Ser 365 370 375	1217
GAC AAA TCT AAA ATC AAT TAACTTTTCT TCCTAGCTCT ATTAGGAATA Asp Lys Ser Lys Ile Asn 380	1265
AACACTECTT CTCITTTTACT TATTTGTTTC TGCTTTAAGT TTAAAATGTA CTCGTGAAAC	1325
CTTTTTTTTA TTAATGTATT TACGTTAC	1353

FIG.3c

RECTIFIED SHEET (RULE 91)
ISA/EP

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Met	Val	Val	Ala	Met	Asp	Gln	Arg	Ser	Asn	Val	Asn	Gly	Asp	Ser	Gly	1	5	10	15
Ala	Arg	Lys	Glu	Glu	Gly	Phe	Asp	Pro	Ser	Ala	Gln	Pro	Pro	Phe	Lys	20	25	30	
Ile	Gly	Asp	Ile	Arg	Ala	Ala	Ile	Pro	Lys	His	Cys	Trp	Val	Lys	Ser	35	40	45	
Pro	Leu	Arg	Ser	Met	Ser	Tyr	Val	Thr	Arg	Asp	Ile	Phe	Ala	Val	Ala	50	55	60	
Ala	Leu	Ala	Met	Ala	Ala	Val	Tyr	Phe	Asp	Ser	Trp	Phe	Leu	Trp	Pro	65	70	75	80
Leu	Tyr	Trp	Val	Ala	Gln	Gly	Thr	Leu	Phe	Trp	Ala	Ile	Phe	Val	Leu	85	90	95	
Gly	His	Asp	Cys	Gly	His	Gly	Ser	Phe	Ser	Asp	Ile	Pro	Leu	Leu	Asn	100	105	110	
Ser	Val	Val	Gly	His	Ile	Leu	His	Ser	Phe	Ile	Leu	Val	Pro	Tyr	His	115	120	125	
Gly	Trp	Arg	Ile	Ser	His	Arg	Thr	His	His	Gln	Asn	His	Gly	His	Val	130	135	140	
Glu	Asn	Asp	Glu	Ser	Trp	Val	Pro	Leu	Pro	Glu	Lys	Leu	Tyr	Lys	Asn	145	150	155	160
Leu	Pro	His	Ser	Thr	Arg	Met	Leu	Arg	Tyr	Thr	Val	Pro	Leu	Pro	Met	165	170	175	
Leu	Ala	Tyr	Pro	Ile	Tyr	Leu	Trp	Tyr	Arg	Ser	Pro	Gly	Lys	Glu	Gly	180	185	190	
Ser	His	Phe	Asn	Pro	Tyr	Ser	Ser	Leu	Phe	Ala	Pro	Ser	Glu	Arg	Lys	195	200	205	
Leu	Ile	Ala	Thr	Ser	Thr	Thr	Cys	Trp	Ser	Ile	Met	Leu	Ala	Thr	Leu	210	215	220	
Val	Tyr	Leu	Ser	Phe	Leu	Val	Asp	Pro	Val	Thr	Val	Leu	Lys	Val	Tyr	225	230	235	240
Gly	Val	Pro	Tyr	Ile	Ile	Phe	Val	Met	Trp	Leu	Asp	Ala	Val	Thr	Tyr	245	250	255	

FIG.3d

RECTIFIED SHEET (RULE 91)
ISA/EP

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Leu His His His Gly His Asp Glu Lys Leu Pro Trp Tyr Arg Gly Lys
 260 265 270

Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Ile Asp Arg Asp Tyr
 275 280 285

Gly Ile Phe Asn Asn Ile His His Asp Ile Gly Thr His Val Ile His
 290 295 300

His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Asp Ala Thr Arg
 305 310 315 320

Ala Ala Lys His Val Leu Gly Arg Tyr Tyr Arg Glu Pro Lys Thr Ser
 325 330 335

Gly Ala Ile Pro Ile His Leu Val Glu Ser Leu Val Ala Ser Ile Lys
 340 345 350

Lys Asp His Tyr Val Ser Asp Thr Gly Asp Ile Val Phe Tyr Glu Thr
 355 360 365

Asp Pro Asp Leu Tyr Val Tyr Ala Ser Asp Lys Ser Lys Ile Asn
 370 375 380

FIG.3e

RECTIFIED SHEET (RULE 91)
 ISA/EP

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	10	20	30	40	50	60
BND3.AMI	RSNVNGDSGARKEEGFDPSAQPPFKIGDIRAAIPKHCWKSPLRSMSYVTRDIFAVAALA					
					
DESA.AMI	MTATIPPLTPTVTPSNPDRPIADLKLQDIKTLPKCEFEKKASKAWASVLI TLGAI AVGY					
	10	20	30	40	50	60
	70	80	90	100	110	120
BND3.AMI	MAAVYFDSWFLWPLYWVAQGTLFWAIFVLGHDCGHGSFSDIPLLNSVVGHILHSFILVPY					
	.. .X.					
DESA.AMI	LGIIYL-PWYCLPITWIWTGTALTGAFVVGHDGHRSAKKRWVNDLVGHI AF APLIYPF					
	70	80	90	100	110	
	130	140	150	160	170	180
BND3.AMI	HGWRI SHRTHQHNGHVENDESWVPLPEKLYKNLPHSTRMLRYTVPLPH-LAYPIYLWYR					
					
DESA.AMI	HSWRLLHDHHLHTNKIEVDNAWDFWSVEAFQASPAIVRLFYRAIRGPFWWTGSIFHW-					
	120	130	140	150	160	170
	190	200	210	220	230	240
BND3.AMI	SPGKEGSHFNPYSSLFAPSERKLIATSTTCWSIMLATLVYLSFLVDP-V-TVLKVYGVY					
 V:					
DESA.AMI	—SLMHFK—LSNFAQRDRNKVKLSIAV-VFLFAAIAFPALIIITGVWGFVKFWLMPW					
	180	190	200	210	220	230
	250	260	270	280	290	300
BND3.AMI	IIFVMWLDVAVTYLHHHGHDEKLPWYRGKEWSYLRGGL-TTIDRDYGFNNIH-HDIGTHV					
					
DESA.AMI	LVYHFWMSTFTIVHHTIPEIRF—RPAADWSAAEAQLNGTVHCDYPRWVEVLCHDINVHI					
	240	250	260	270	280	
	310	320	330	340	350	360
BND3.AMI	IHHLFPQIPHYHLVDATRAAKHVLGRYYREPKTSGAIP IHLVESLVASIKKDHVVS DTGD					
					
DESA.AMI	PHHLSVAIPSYNRLAHGSLKENWGPFLYERTFNWQLMQQISGQCHLYDPEHGYRTFGSL					
	290	300	310	320	330	340
BND3.AMI	IVF					
DESA.AMI	KKV					
	350					

FIG.4

RECTIFIED SHEET (RULE 91)
ISA/EP

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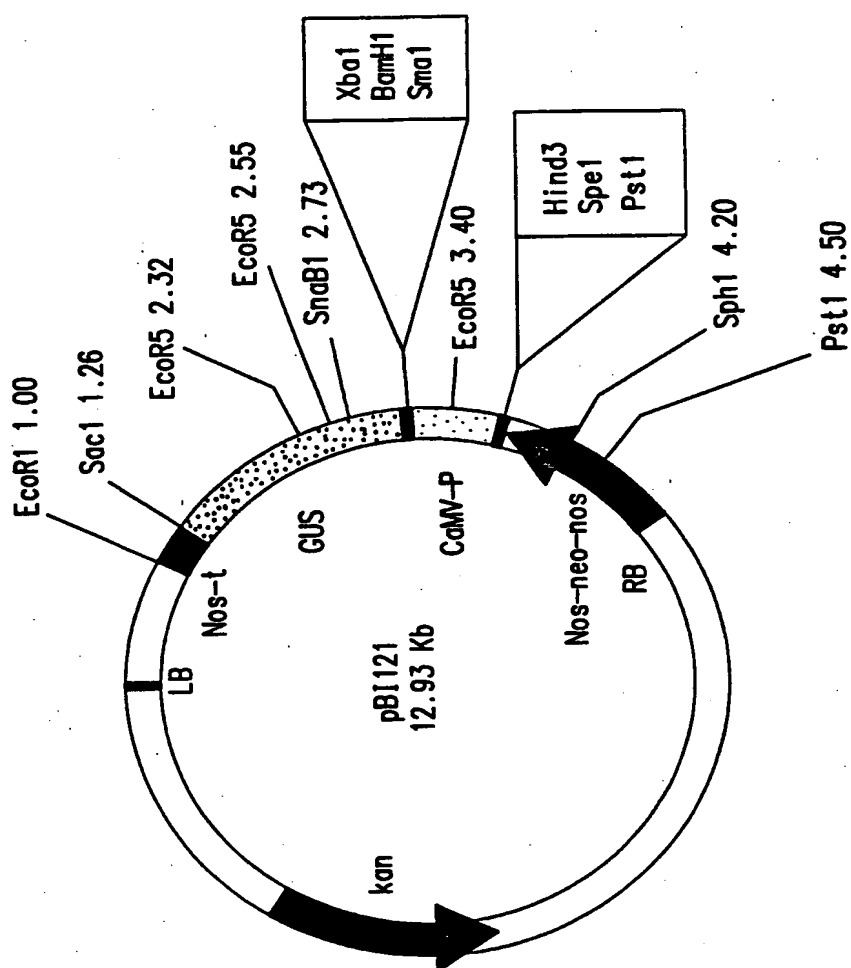


FIG.5

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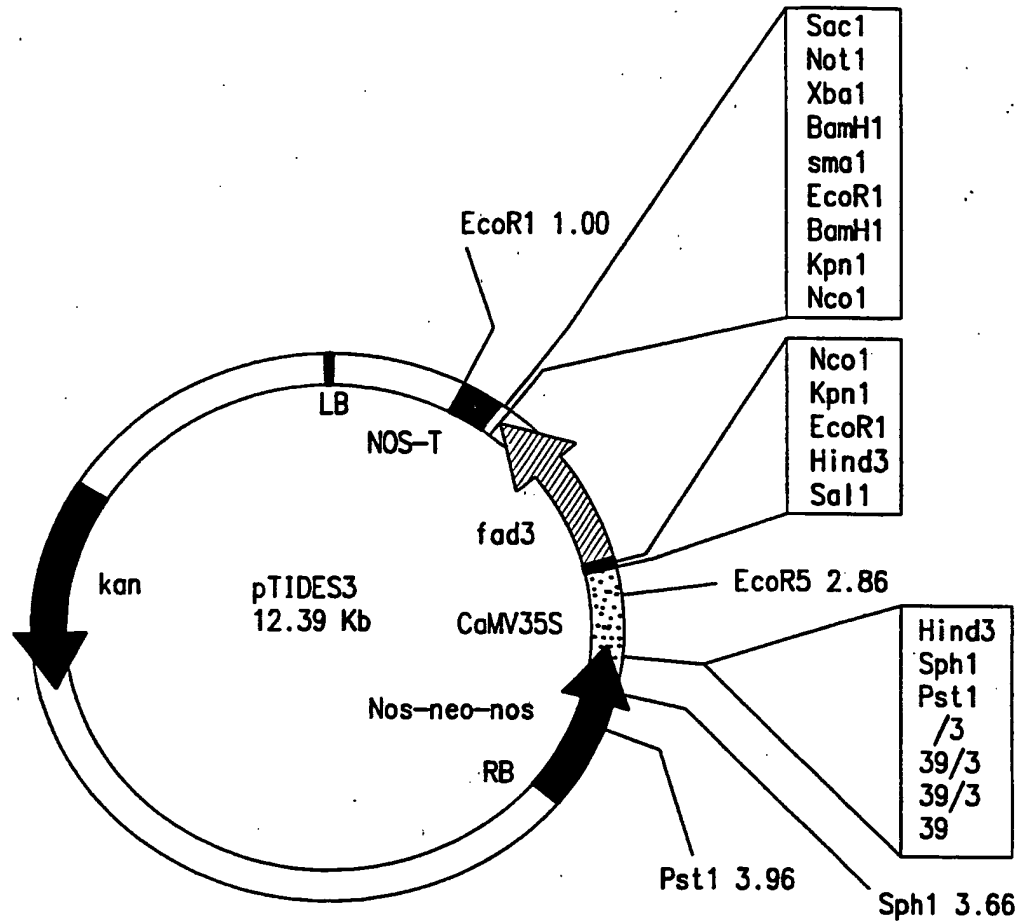


FIG.6

RECTIFIED SHEET (RULE 91)
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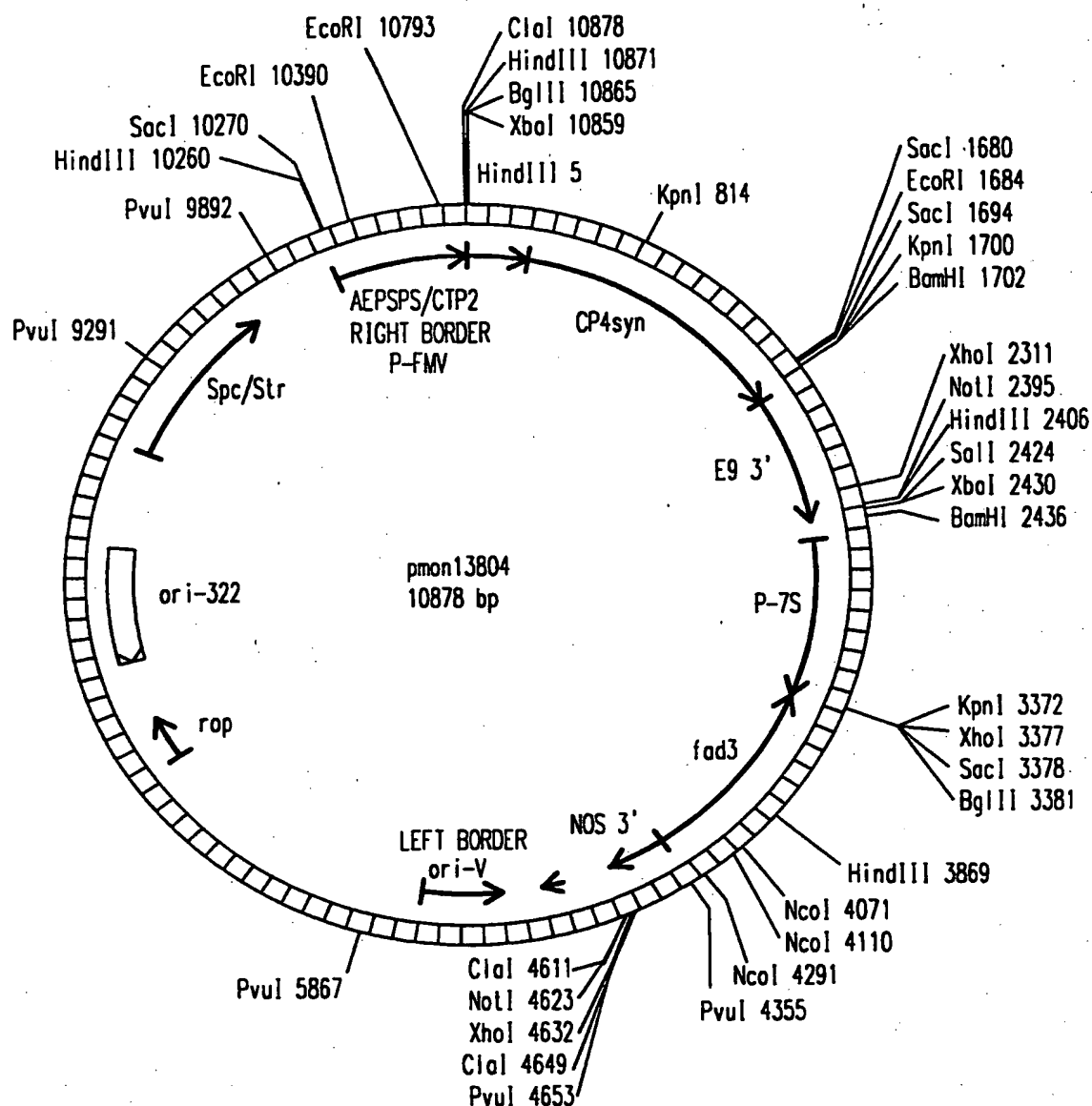


FIG.7

RECTIFIED SHEET (RULE 91)
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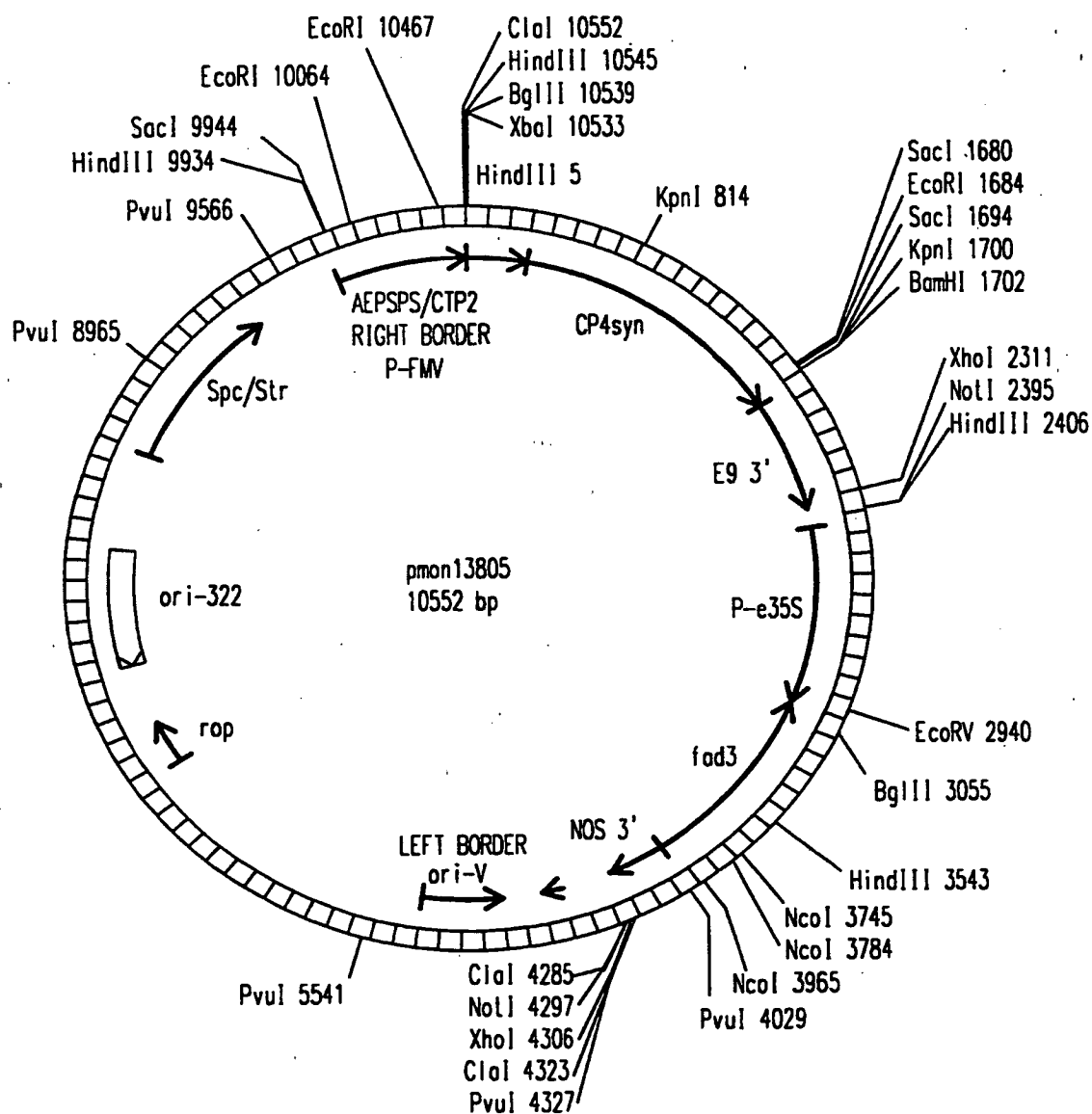


FIG.8

RECTIFIED SHEET (RULE 91)
ISA/EP

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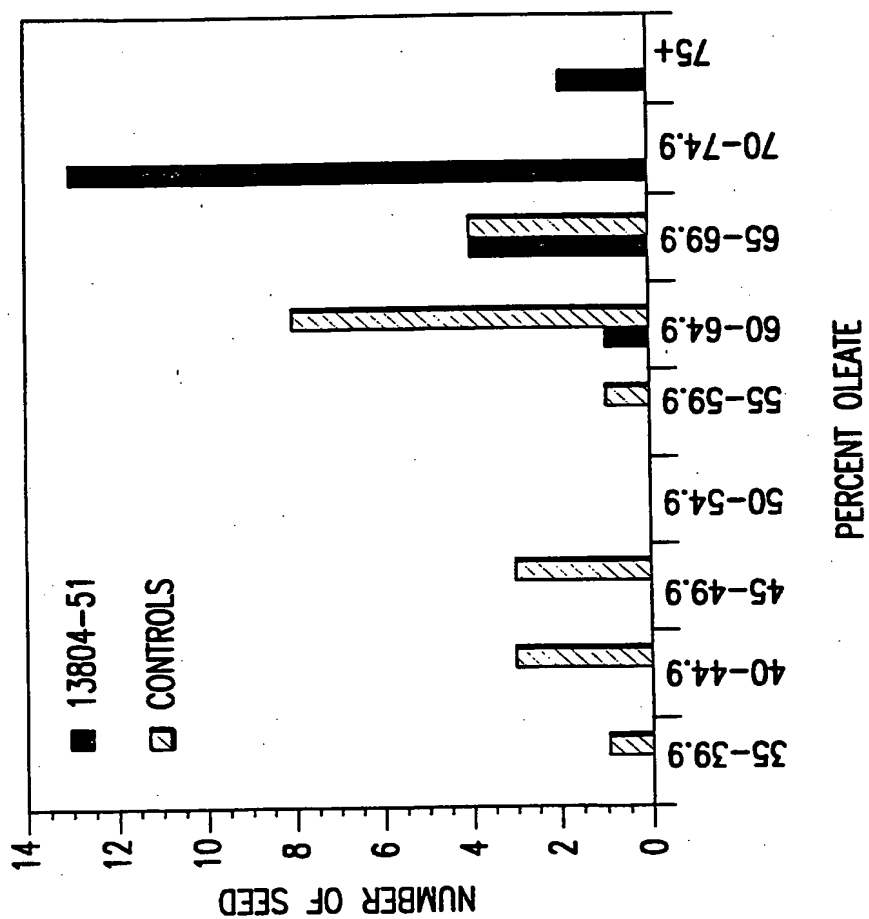


FIG. 9a

RECTIFIED SHEET (RULE 91)
ISA/EP

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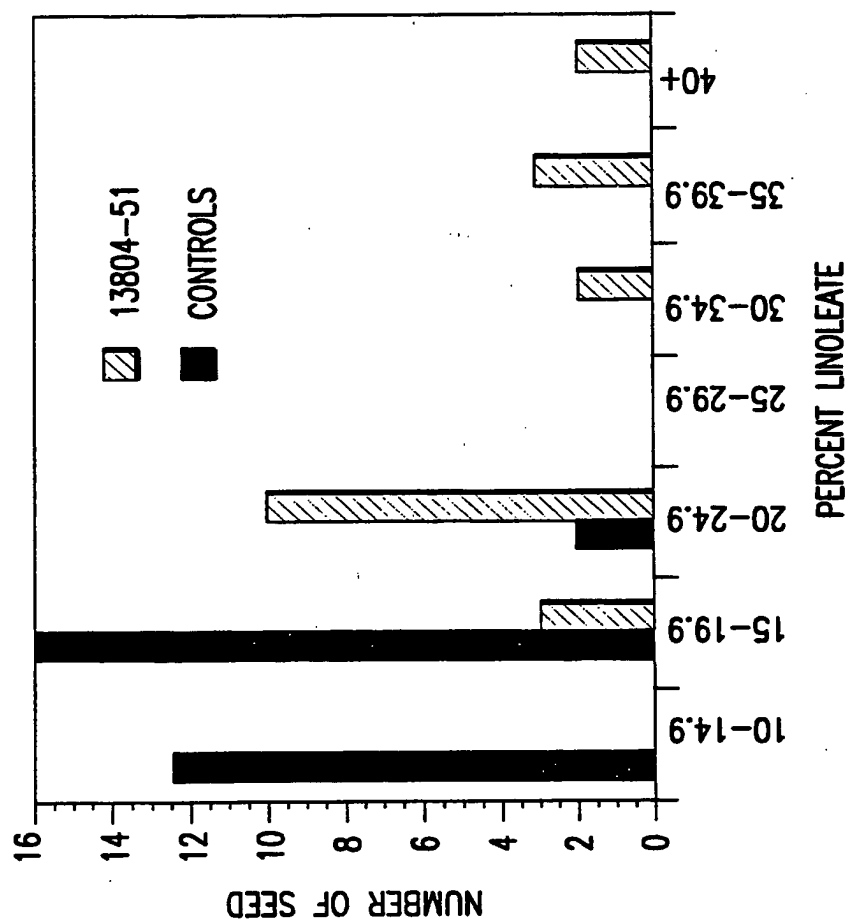


FIG.9b

RECTIFIED SHEET (RULE 91)
ISA/EP

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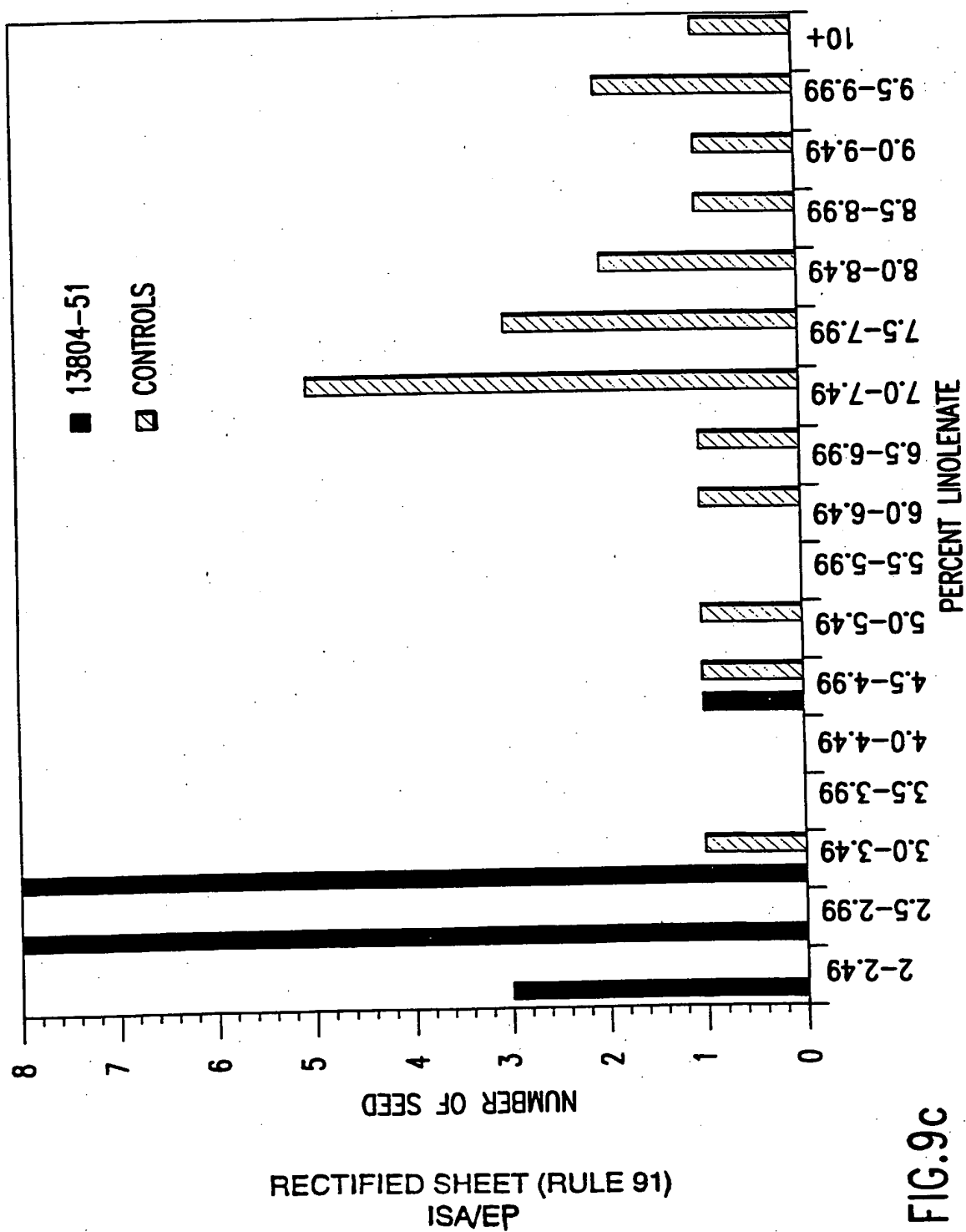


FIG. 9c

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GGAAAACACA AGTTTCTCTC ACACACATTA TCTCTTTCTC TATTACCACC ACTCATTCAT	60
AACAGAAACC CACCAAAAAA TAAAAAGAGA GACTTTTCAC TCTGGGGAGA GAGCTCAAGT	120
TCTA ATG GCG AAC TTG GTC TTA TCA GAA TGT GGT ATA CGA CCT CTC CCC Met Ala Asn Leu Val Leu Ser Glu Cys Gly Ile Arg Pro Leu Pro 1 5 10 15	169
AGA ATC TAC ACA ACA CCC AGA TCC AAT TTC CTC TCC AAC AAC AAC AAA Arg Ile Tyr Thr Thr Pro Arg Ser Asn Phe Leu Ser Asn Asn Asn Lys 20 25 30	217
TTC AGA CCA TCA CTT TCT TCT TCT TCT TAC AAA ACA TCA TCA TCT CCT Phe Arg Pro Ser Leu Ser Ser Ser Ser Tyr Lys Thr Ser Ser Ser Pro 35 40 45	265
CTG TCT TTT GGT CTG AAT TCA CGA GAT GGG TTC ACG AGG AAT TGG GCG Leu Ser Phe Gly Leu Asn Ser Arg Asp Gly Phe Thr Arg Asn Trp Ala 50 55 60	313
TTG AAT GTG AGC ACA CCA TTA ACG ACA CCA ATA TTT GAG GAG TCT CCA Leu Asn Val Ser Thr Pro Leu Thr Thr Pro Ile Phe Glu Glu Ser Pro 65 70 75	361
TTG GAG GAA GAT AAT AAA CAG AGA TTC GAT CCA GGT GCG CCT CCT CCG Leu Glu Glu Asp Asn Lys Gln Arg Phe Asp Pro Gly Ala Pro Pro Pro 80 85 90 95	409
TTC AAT TTA GCT GAT ATT AGA GCA GCT ATA CCT AAG CAT TGT TGG GTT Phe Asn Leu Ala Asp Ile Arg Ala Ala Ile Pro Lys His Cys Trp Val 100 105 110	457
AAG AAT CCA TGG AAG TCT TTG AGT TAT GTC GTC AGA GAC GTC GCT ATC Lys Asn Pro Trp Lys Ser Leu Ser Tyr Val Val Arg Asp Val Ala Ile 115 120 125	505
GTC TTT GCA TTG GCT GCT GGA GCT GCT TAC CTC AAC AAT TGG ATT GTT Val Phe Ala Leu Ala Ala Gly Ala Ala Tyr Leu Asn Asn Trp Ile Val 130 135 140	553

FIG.10a

RECTIFIED SHEET (RULE 91)
ISAVEP

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TGG CCT CTC TAT TGG CTC GCT CAA GGA ACC ATG TTT TGG GCT CTC TTT Trp Pro Leu Tyr Trp Leu Ala Gln Gly Thr Met Phe Trp Ala Leu Phe 145 150 155	601
GTT CTT GGT CAT GAC TGT GGA CAT GGT AGT TTC TCA AAT GAT CCG AAG Val Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asn Asp Pro Lys 160 165 170 175	649
TTG AAC AGT GTG GTC GGT CAT CTT CTT CAT TCC TCA ATT CTG GTC CCA Leu Asn Ser Val Val Gly His Leu Leu His Ser Ser Ile Leu Val Pro 180 185 190	697
TAC CAT GGC TGG AGA ATT AGT CAC AGA ACT CAC CAC CAG AAC CAT GGA Tyr His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly 195 200 205	745
CAT GTT GAG AAT GAC GAA TCT TGG CAT CCT ATG TCT GAG AAA ATC TAC His Val Glu Asn Asp Glu Ser Trp His Pro Met Ser Glu Lys Ile Tyr 210 215 220	793
AAT ACT TTG GAC AAG CCG ACT AGA TTC TTT AGA TTT ACA CTG CCT CTC Asn Thr Leu Asp Lys Pro Thr Arg Phe Phe Arg Phe Thr Leu Pro Leu 225 230 235	841
GTG ATG CTT GCA TAC CCT TTC TAC TTG TGG GCT CGA AGT CCG GGG AAA Val Met Leu Ala Tyr Pro Phe Tyr Leu Trp Ala Arg Ser Pro Gly Lys 240 245 250 255	889
AAG GGT TCT CAT TAC CAT CCA GAC AGT GAC TTG TTC CTC CCT AAA GAG Lys Gly Ser His Tyr His Pro Asp Ser Asp Leu Phe Leu Pro Lys Glu 260 265 270	937
AGA AAG GAT GTC CTC ACT TCT ACT GCT TGT TGG ACT GCA ATG GCT GCT Arg Lys Asp Val Leu Thr Ser Thr Ala Cys Trp Thr Ala Met Ala Ala 275 280 285	985
CTG CTT GTT TGT CTC AAC TTC ACA ATC GGT CCA ATT CAA ATG CTC AAA Leu Leu Val Cys Leu Asn Phe Thr Ile Gly Pro Ile Gln Met Leu Lys 290 295 300	1033

FIG.10b

RECTIFIED SHEET (RULE 91)
ISA/EP

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CTT TAT GGA ATT CCT TAC TGG ATA AAT GTA ATG TGG TTG GAC TTT GTG Leu Tyr Gly Ile Pro Tyr Trp Ile Asn Val Met Trp Leu Asp Phe Val 305 310 315	1081
ACT TAC CTG CAT CAC CAT GGT CAT GAA GAT AAG CTT CCT TGG TAC CGT Thr Tyr Leu His His His Gly His Glu Asp Lys Leu Pro Trp Tyr Arg 320 325 330 335	1129
GGC AAG GAG TGG AGT TAC CTG AGA GGA GGA CTT ACA ACA TTG GAT CGT Gly Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Leu Asp Arg 340 345 350	1177
GAC TAC GGA TTG ATC AAT AAC ATC CAT CAT GAT ATT GGA ACT CAT GTG Asp Tyr Gly Leu Ile Asn Asn Ile His His Asp Ile Gly Thr His Val 355 360 365	1225
ATA CAT CAT CTT TTC CCG CAG ATC CCA CAT TAT CAT CTA GTA GAA GCA Ile His His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Glu Ala 370 375 380	1273
ACA GAA GCA GCT AAA CCA GTA TTA GGG AAG TAT TAC AGG GAG CCT GAT Thr Glu Ala Ala Lys Pro Val Leu Gly Lys Tyr Tyr Arg Glu Pro Asp 385 390 395	1321
AAG TCT GGA CCG TTG CCA TTA CAT TTA CTG GAA ATT CTA GCG AAA AGT Lys Ser Gly Pro Leu Pro Leu His Leu Leu Glu Ile Leu Ala Lys Ser 400 405 410 415	1369
ATA AAA GAA GAT CAT TAC GTG AGC GAC GAA GGA GAA GTT GTA TAC TAT Ile Lys Glu Asp His Tyr Val Ser Asp Glu Gly Glu Val Val Tyr Tyr 420 425 430	1417
AAA GCA GAT CCA AAT CTC TAT GGA GAG GTC AAA GTA AGA GCA GAT TGAAATGAAG Lys Ala Asp Pro Asn Leu Tyr Gly Glu Val Lys Val Arg Ala Asp 435 440 445	1472
CAGGCTTGAG ATTGAAGTTT TTTCTATTTC AGACCAGCTG ATTTTTTGCT TACTGTATCA	1532
ATTTATTGTG TCACCCACCA GAGAGTTAGT ATCTCTGAAT ACGATCGATC AGATGGAAC	1592
AACAAATTTG TTTGCGATAC TGAAGCTATA TATACCATAA AAAAAAAAAA AAA	1645

FIG.10c

RECTIFIED SHEET (RULE 91)
ISA/EP

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Met Ala Asn Leu Val Leu Ser Glu Cys Gly Ile Arg Pro Leu Pro Arg
 1 5 10 15
 Ile Tyr Thr Thr Pro Arg Ser Asn Phe Leu Ser Asn Asn Asn Lys Phe
 20 25 30
 Arg Pro Ser Leu Ser Ser Ser Ser Tyr Lys Thr Ser Ser Ser Pro Leu
 35 40 45
 Ser Phe Gly Leu Asn Ser Arg Asp Gly Phe Thr Arg Asn Trp Ala Leu
 50 55 60
 Asn Val Ser Thr Pro Leu Thr Thr Pro Ile Phe Glu Glu Ser Pro Leu
 65 70 75 80
 Glu Glu Asp Asn Lys Gln Arg Phe Asp Pro Gly Ala Pro Pro Pro Phe
 85 90 95
 Asn Leu Ala Asp Ile Arg Ala Ala Ile Pro Lys His Cys Trp Val Lys
 100 105 110
 Asn Pro Trp Lys Ser Leu Ser Tyr Val Val Arg Asp Val Ala Ile Val
 115 120 125
 Phe Ala Leu Ala Ala Gly Ala Ala Tyr Leu Asn Asn Trp Ile Val Trp
 130 135 140
 Pro Leu Tyr Trp Leu Ala Gln Gly Thr Met Phe Trp Ala Leu Phe Val
 145 150 155 160
 Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asn Asp Pro Lys Leu
 165 170 175
 Asn Ser Val Val Gly His Leu Leu His Ser Ser Ile Leu Val Pro Tyr
 180 185 190
 His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly His
 195 200 205
 Val Glu Asn Asp Glu Ser Trp His Pro Met Ser Glu Lys Ile Tyr Asn
 210 215 220

FIG.11a
 RECTIFIED SHEET (RULE 91)
 ISA/EP

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Thr	Leu	Asp	Lys	Pro	Thr	Arg	Phe	Phe	Arg	Phe	Thr	Leu	Pro	Leu	Val	225	230	235	240
Met	Leu	Ala	Tyr	Pro	Phe	Tyr	Leu	Trp	Ala	Arg	Ser	Pro	Gly	Lys	Lys	245	250	255	
Gly	Ser	His	Tyr	His	Pro	Asp	Ser	Asp	Leu	Phe	Leu	Pro	Lys	Glu	Arg	260	265	270	
Lys	Asp	Val	Leu	Thr	Ser	Thr	Ala	Cys	Trp	Thr	Ala	Met	Ala	Ala	Leu	275	280	285	
Leu	Val	Cys	Leu	Asn	Phe	Thr	Ile	Gly	Pro	Ile	Gln	Met	Leu	Lys	Leu	290	295	300	
Tyr	Gly	Ile	Pro	Tyr	Trp	Ile	Asn	Val	Met	Trp	Leu	Asp	Phe	Val	Thr	305	310	315	320
Tyr	Leu	His	His	His	Gly	His	Glu	Asp	Lys	Leu	Pro	Trp	Tyr	Arg	Gly	325	330	335	
Lys	Glu	Trp	Ser	Tyr	Leu	Arg	Gly	Gly	Leu	Thr	Thr	Leu	Asp	Arg	Asp	340	345	350	
Tyr	Gly	Leu	Ile	Asn	Asn	Ile	His	His	Asp	Ile	Gly	Thr	His	Val	Ile	355	360	365	
His	His	Leu	Phe	Pro	Gln	Ile	Pro	His	Tyr	His	Leu	Val	Glu	Ala	Thr	370	375	380	
Glu	Ala	Ala	Lys	Pro	Val	Leu	Gly	Lys	Tyr	Tyr	Arg	Glu	Pro	Asp	Lys	385	390	395	400
Ser	Gly	Pro	Leu	Pro	Leu	His	Leu	Leu	Glu	Ile	Leu	Ala	Lys	Ser	Ile	405	410	415	
Lys	Glu	Asp	His	Tyr	Val	Ser	Asp	Glu	Gly	Glu	Val	Val	Tyr	Tyr	Lys	420	425	430	
Ala	Asp	Pro	Asn	Leu	Tyr	Gly	Glu	Val	Lys	Val	Arg	Ala	Asp			435	440	445	

FIG.11b

RECTIFIED SHEET (RULE 91)
ISA/EP

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AGAGAGTGCA AATAGAACGA CAGAGACTTT TTCCTCTTTT CTTCCTGGGA AGAGGCTCCA	60
ATG GCG AGC TCG GTT TTA TCA GAA TGT GGT TTT AGA CCT CTC CCC AGA Met Ala Ser Ser Val Leu Ser Glu Cys Gly Phe Arg Pro Leu Pro Arg	108
1 5 10 15	
TTC TAC CCT AAA CAC ACA ACC TCT TTT GCC TCT AAC CCT AAA CCC ACT Phe Tyr Pro Lys His Thr Thr Ser Phe Ala Ser Asn Pro Lys Pro Thr	156
20 25 30	
TTC AAA TTC AAT CCA CCA CTT AAA CCT CCT TCT TCT CTT CTC AAT TCC Phe Lys Phe Asn Pro Pro Leu Lys Pro Pro Ser Ser Leu Leu Asn Ser	204
35 40 45	
CGA TAT GGA TTC TAC TCT AAA ACC AGG AAC TGG GCA TTG AAT GTG GCA Arg Tyr Gly Phe Tyr Ser Lys Thr Arg Asn Trp Ala Leu Asn Val Ala	252
50 55 60	
ACA CCT TTA ACA ACT CTT CAG TCT CCA TCC GAG GAA GAC ACG GAG AGA Thr Pro Leu Thr Thr Leu Gln Ser Pro Ser Glu Glu Asp Thr Glu Arg	300
65 70 75 80	
TTC GAC CCA GGT GCG CCT CCT CCC TTC AAT TTG GCG GAT ATA AGA GCA Phe Asp Pro Gly Ala Pro Pro Pro Phe Asn Leu Ala Asp Ile Arg Ala	348
85 90 95	
GCC ATA CCT AAG CAT TGT TGG GTT AAG AAT CCA TGG ATG TCT ATG AGT Ala Ile Pro Lys His Cys Trp Val Lys Asn Pro Trp Met Ser Met Ser	396
100 105 110	
TAT GTT GTC AGA GAT GTT GCT ATC GTC TTT GGA TTG GCT GCT GTT GCT Tyr Val Val Arg Asp Val Ala Ile Val Phe Gly Leu Ala Ala Val Ala	444
115 120 125	
GCT TAC TTC AAC AAT TGG CTT CTC TGG CCT CTC TAC TGG TTC GCT CAA Ala Tyr Phe Asn Asn Trp Leu Leu Trp Pro Leu Tyr Trp Phe Ala Gln	492
130 135 140	
GGA ACC ATG TTC TGG GCT CTC TTT GTC CTT GGC CAT GAC TGC GGA CAT Gly Thr Met Phe Trp Ala Leu Phe Val Leu Gly His Asp Cys Gly His	540
145 150 155 160	

FIG.12a
RECTIFIED SHEET (RULE 91)
ISA/EP

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GGT AGC TTC TCG AAT GAT CCG AGG CTG AAC AGT GTG GCT GGT CAT CTT Gly Ser Phe Ser Asn Asp Pro Arg Leu Asn Ser Val Ala Gly His Leu 165 170 175	588
CTT CAT TCC TCA ATT CTG GTC CCT TAC CAT GGC TGG AGG ATT AGC CAC Leu His Ser Ser Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His 180 185 190	636
AGA ACT CAC CAC CAG AAC CAT GGT CAT GTC GAG AAT GAC GAA TCA TGG Arg Thr His His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp 195 200 205	684
CAT CCT TTG CCT GAA AGC ATC TAC AAG AAT TTG GAA AAG ACG ACT CAA His Pro Leu Pro Glu Ser Ile Tyr Lys Asn Leu Glu Lys Thr Thr Gln 210 215 220	732
ATG TTT AGG TTT ACA CTG CCT TTT CCA ATG CTC GCA TAC CCT TTC TAC Met Phe Arg Phe Thr Leu Pro Phe Pro Met Leu Ala Tyr Pro Phe Tyr 225 230 235 240	780
TTG TGG AAC AGA AGT CCA GGG AAA CAA GGT TCT CAT TAT CAT CCG GAC Leu Trp Asn Arg Ser Pro Gly Lys Gln Gly Ser His Tyr His Pro Asp 245 250 255	828
AGT GAC TTG TTT CTT CCA AAA GAG AAG AAA GAT GTT CTG ACA TCA ACT Ser Asp Leu Phe Leu Pro Lys Glu Lys Lys Asp Val Leu Thr Ser Thr 260 265 270	876
GCC TGT TGG ACT GCA ATG GCT GCT TTG CTT GTT TGT CTC AAC TTT GTC Ala Cys Trp Thr Ala Met Ala Ala Leu Leu Val Cys Leu Asn Phe Val 275 280 285	924
ATG GGT CCA ATC CAG ATG CTC AAA CTA TAT GGC ATC CCT TAT TGG ATA Met Gly Pro Ile Gln Met Leu Lys Leu Tyr Gly Ile Pro Tyr Trp Ile 290 295 300	972
TTT GTA ATG TGG TTG GAC TTC GTC ACT TAC TTG CAC CAC CAT GGA CAT Phe Val Met Trp Leu Asp Phe Val Thr Tyr Leu His His His Gly His 305 310 315 320	1020

FIG.12b
RECTIFIED SHEET (RULE 91)
ISA/EP

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GAA GAC AAG CTC CCT TGG TAT CGT GGA AAG GAA TGG AGT TAC CTG AGA Glu Asp Lys Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg 325 330 335	1068
GGA GGG CTC ACA ACA TTA GAT CGT GAC TAC GGA TGG ATC AAT AAC ATC Gly Gly Leu Thr Thr Leu Asp Arg Asp Tyr Gly Trp Ile Asn Asn Ile 340 345 350	1116
CAC CAC GAT ATT GGA ACT CAT GTG ATA CAT CAT CTT TTC CCG CAG ATC His His Asp Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile 355 360 365	1164
CCA CAT TAT CAT CTA GTA GAA GCA ACA GAA GCA GCT AAA CCA GTA CTA Pro His Tyr His Leu Val Glu Ala Thr Glu Ala Ala Lys Pro Val Leu 370 375 380	1212
GGA AAG TAC TAC AGA GAA CCG AAA AAC TCT GGA CCT CTG CCA CTT CAC Gly Lys Tyr Tyr Arg Glu Pro Lys Asn Ser Gly Pro Leu Pro Leu His 385 390 395 400	1260
TTA CTG GGA AGC CTC ATA AAG AGT ATG AAA CAA GAC CAT TTC GTA AGC Leu Leu Gly Ser Leu Ile Lys Ser Met Lys Gln Asp His Phe Val Ser 405 410 415	1308
GAT ACA GGA GAT GTC GTG TAC TAT GAG GCA GAT CCA AAA CTC AAT GGA Asp Thr Gly Asp Val Val Tyr Tyr Glu Ala Asp Pro Lys Leu Asn Gly 420 425 430	1356
CAA AGA ACA TGAGGACATA CTGCAGTGAA CCAGGCAGAC AAGTTACATA Gln Arg Thr 435	1405
AATTCATCTT GGCCATTCA TTATGTTCTT TTTGTTTTGG TGTAAGCCT TTTCGAGATT	1465
AAAAAAGCAT TAATTTGTAG AAACCTGTGG TAAACTCTC GATCAAATGA AATAAGATAT	1525

FIG.12c

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Met	Ala	Ser	Ser	Val	Leu	Ser	Glu	Cys	Gly	Phe	Arg	Pro	Leu	Pro	Arg	1	5	10	15
Phe	Tyr	Pro	Lys	His	Thr	Thr	Ser	Phe	Ala	Ser	Asn	Pro	Lys	Pro	Thr	20	25	30	
Phe	Lys	Phe	Asn	Pro	Pro	Leu	Lys	Pro	Pro	Ser	Ser	Leu	Leu	Asn	Ser	35	40	45	
Arg	Tyr	Gly	Phe	Tyr	Ser	Lys	Thr	Arg	Asn	Trp	Ala	Leu	Asn	Val	Ala	50	55	60	
Thr	Pro	Leu	Thr	Thr	Leu	Gln	Ser	Pro	Ser	Glu	Glu	Asp	Thr	Glu	Arg	65	70	75	80
Phe	Asp	Pro	Gly	Ala	Pro	Pro	Pro	Phe	Asn	Leu	Ala	Asp	Ile	Arg	Ala	85	90	95	
Ala	Ile	Pro	Lys	His	Cys	Trp	Val	Lys	Asn	Pro	Trp	Met	Ser	Met	Ser	100	105	110	
Tyr	Val	Val	Arg	Asp	Val	Ala	Ile	Val	Phe	Gly	Leu	Ala	Ala	Val	Ala	115	120	125	
Ala	Tyr	Phe	Asn	Asn	Trp	Leu	Leu	Trp	Pro	Leu	Tyr	Trp	Phe	Ala	Gln	130	135	140	
Gly	Thr	Met	Phe	Trp	Ala	Leu	Phe	Val	Leu	Gly	His	Asp	Cys	Gly	His	145	150	155	160
Gly	Ser	Phe	Ser	Asn	Asp	Pro	Arg	Leu	Asn	Ser	Val	Ala	Gly	His	Leu	165	170	175	
Leu	His	Ser	Ser	Ile	Leu	Val	Pro	Tyr	His	Gly	Trp	Arg	Ile	Ser	His	180	185	190	
Arg	Thr	His	His	Gln	Asn	His	Gly	His	Val	Glu	Asn	Asp	Glu	Ser	Trp	195	200	205	
His	Pro	Leu	Pro	Glu	Ser	Ile	Tyr	Lys	Asn	Leu	Glu	Lys	Thr	Thr	Gln	210	215	220	

FIG.13a

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Met Phe Arg Phe Thr Leu Pro Phe Pro Met Leu Ala Tyr Pro Phe Tyr
 225 230 235 240
 Leu Trp Asn Arg Ser Pro Gly Lys Gln Gly Ser His Tyr His Pro Asp
 245 250 255
 Ser Asp Leu Phe Leu Pro Lys Glu Lys Lys Asp Val Leu Thr Ser Thr
 260 265 270
 Ala Cys Trp Thr Ala Met Ala Ala Leu Leu Val Cys Leu Asn Phe Val
 275 280 285
 Met Gly Pro Ile Gln Met Leu Lys Leu Tyr Gly Ile Pro Tyr Trp Ile
 290 295 300
 Phe Val Met Trp Leu Asp Phe Val Thr Tyr Leu His His His Gly His
 305 310 315 320
 Glu Asp Lys Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg
 325 330 335
 Gly Gly Leu Thr Thr Leu Asp Arg Asp Tyr Gly Trp Ile Asn Asn Ile
 340 345 350
 His His Asp Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile
 355 360 365
 Pro His Tyr His Leu Val Glu Ala Thr Glu Ala Ala Lys Pro Val Leu
 370 375 380
 Gly Lys Tyr Tyr Arg Glu Pro Lys Asn Ser Gly Pro Leu Pro Leu His
 385 390 395 400
 Leu Leu Gly Ser Leu Ile Lys Ser Met Lys Gln Asp His Phe Val Ser
 405 410 415
 Asp Thr Gly Asp Val Val Tyr Tyr Glu Ala Asp Pro Lys Leu Asn Gly
 420 425 430
 Gln Arg Thr
 435

FIG.13b

RECTIFIED SHEET (RULE 91)
ISA/EP

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 94/01321

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/82 C12N15/53 C12N15/11 C12N5/10 A01H5/00
C11B1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N A01H C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE vol. 258 , 20 November 1992 , LANCASTER, PA US pages 1353 - 1355 ARONDEL, V., ET AL. 'Map-based cloning of a gene controlling omega-3 fatty acid desaturation in Arabidopsis'	37
Y	see the whole document	1,2,4,8, 9,11,17, 18,22, 23,25, 29,30, 32,38
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☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

1 June 1994

Date of mailing of the international search report

14 -06- 1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US,A,5 057 419 (MARTIN) 15 October 1991 see column 6, line 40 - column 6, line 66 see column 9, line 25 - column 10, line 58 ---	20,40 1,2,4,8, 9,11,17, 18,22, 23,25, 29,30, 32,38
Y	WO,A,91 13972 (CALGENE) 19 September 1991 see the whole document ---	1,2,4,8, 9,11,17, 18,22, 23,25, 29,30, 32,38
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 268, no. 32 , 15 November 1993 , BALTIMORE, MD US pages 24099 - 24105 IBA, K., ET AL. 'A gene encoding a chloroplast omega-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy numbers of the fad7 mutant of Arabidopsis thaliana' see the whole document ---	1,2,5, 22,23, 26,37
P,X	PLANT PHYSIOLOGY. vol. 103 , October 1993 , ROCKVILLE, MD, USA. pages 467 - 476 YADAV, N.S., ET AL. 'Cloning of higher plant omega-3- fatty acid desaturases' see the whole document ---	1,2,17, 22,23,37
P,X	WO,A,93 11245 (DU PONT) 10 June 1993 see the whole document ---	1,2,8,9, 17,22, 23,29, 30,37,38
P,X	WO,A,93 06712 (RHONE-POULENC AGROCHIMIE) 15 April 1993 ---	1,2,22, 23,37
A	PLANT PHYSIOLOGY. vol. 100 , 1992 , ROCKVILLE, MD, USA. pages 894 - 901 POLASHOCK, J.J., ET AL. 'Expression of the yeast delta-9 fatty acid desaturase in Nicotina tabacum' see the whole document --- -/--	1,22

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 94/01321

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANN. REV. PLANT PHYSIOL. PLANT MOL. BIOL. vol. 42 , 1991 pages 467 - 506 BROWSE, J., ET AL. 'Glycerolipid synthesis: Biochemistry and regulation' see the whole document ---	1-40
A	UCLA SYMP. MOL. CELL BIOL, NEW SER. vol. 129 , 1990 pages 301 - 309 BROWSE, J., ET AL. 'Strategies for modifying plant lipid composition' see page 306 ---	1,22
A	NL,A,9 002 130 (STICHTING TECHNISCHE WETENSCHAPPEN UTRECHT) 16 April 1992 see the whole document -----	1-40

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 94/01321

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5057419	15-10-91	NONE	
WO-A-9113972	19-09-91	EP-A- 0472722	04-03-92
WO-A-9311245	10-06-93	AU-A- 3228893	28-06-93
WO-A-9306712	15-04-93	AU-A- 2881292	03-05-93
NL-A-9002130	16-04-92	NONE	

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